

# Middlesex University Research Repository

An open access repository of

Middlesex University research

<http://eprints.mdx.ac.uk>

Christou, Anastasis, Agüera, Ana, Bayona, Josep Maria, Cytryn, Eddie, Fotopoulos, Vasileios, Lambropoulou, Dimitra, Manaia, Célia M., Michael, Costas, Revitt, D. Mike, Schröder, Peter and Fatta-Kassinos, Despo (2017) The potential implications of reclaimed wastewater reuse for irrigation on the agricultural environment: the knowns and unknowns of the fate of antibiotics and antibiotic resistant bacteria and resistance genes – a review. *Water Research*, 123 . pp. 448-467. ISSN 0043-1354 [Article] (doi:10.1016/j.watres.2017.07.004)

Final accepted version (with author's formatting)

This version is available at: <https://eprints.mdx.ac.uk/22228/>

## Copyright:

Middlesex University Research Repository makes the University's research available electronically.

Copyright and moral rights to this work are retained by the author and/or other copyright owners unless otherwise stated. The work is supplied on the understanding that any use for commercial gain is strictly forbidden. A copy may be downloaded for personal, non-commercial, research or study without prior permission and without charge.

Works, including theses and research projects, may not be reproduced in any format or medium, or extensive quotations taken from them, or their content changed in any way, without first obtaining permission in writing from the copyright holder(s). They may not be sold or exploited commercially in any format or medium without the prior written permission of the copyright holder(s).

Full bibliographic details must be given when referring to, or quoting from full items including the author's name, the title of the work, publication details where relevant (place, publisher, date), pagination, and for theses or dissertations the awarding institution, the degree type awarded, and the date of the award.

If you believe that any material held in the repository infringes copyright law, please contact the Repository Team at Middlesex University via the following email address:

[eprints@mdx.ac.uk](mailto:eprints@mdx.ac.uk)

The item will be removed from the repository while any claim is being investigated.

See also repository copyright: re-use policy: <http://eprints.mdx.ac.uk/policies.html#copy>

**1 The potential implications of reclaimed wastewater reuse for irrigation on the agricultural**  
**2 environment: the knowns and unknowns of the fate of antibiotics and antibiotic resistant**  
**3 bacteria and resistance genes – A review**

4

5

6 Anastasis Christou<sup>1\*</sup>, Ana Agüera<sup>2</sup>, Josep Maria Bayona<sup>3</sup>, Eddie Cytryn<sup>4</sup>, Vasileios Fotopoulos<sup>5</sup>,  
7 Dimitra Lambropoulou<sup>6</sup>, Célia M. Manaia<sup>7</sup>, Costas Michael<sup>8</sup>, Mike Revitt<sup>9</sup>, Peter Schröder<sup>10</sup>,  
8 Despo Fatta-Kassinos<sup>8,11\*\*</sup>

9

10

11 <sup>1</sup>Agricultural Research Institute, Ministry of Agriculture, Rural Development and Environment,  
12 P.O. Box 22016, 1516 Nicosia, Cyprus

13 <sup>2</sup>Solar Energy Research Centre (CIESOL), Joint Centre University of Almería-CIEMAT, 04120,  
14 Almería, Spain

15 <sup>3</sup>IDAEA-CSIC, Environmental Chemistry Department, E-08034, Barcelona, Spain

16 <sup>4</sup>Institute of Soil, Water and Environmental Sciences, Volcani Center, Agricultural Research  
17 Organization, P.O Box 15159, Rishon Lezion, Israel

18 <sup>5</sup>Department of Agricultural Sciences, Biotechnology and Food Science, Cyprus University of  
19 Technology, 3603 Lemesos, Cyprus

20 <sup>6</sup>Aristotle University of Thessaloniki, Department of Chemistry, 54124, Thessaloniki, Greece

21 <sup>7</sup>Universidade Católica Portuguesa, CBQF - Centro de Biotecnologia e Química Fina –  
22 Laboratório Associado, Escola Superior de Biotecnologia, Rua Arquiteto Lobão Vital, Apartado  
23 2511, 4202-401 Porto, Portugal

24 <sup>8</sup>NIREAS-International Water Research Center, University of Cyprus, P.O. Box 20537, 1678  
25 Nicosia, Cyprus

26 <sup>9</sup>Middlesex University, Department of Natural Sciences, NW4 4BT London, United Kingdom

27 <sup>10</sup>Helmholtz Zentrum München, German Research Center for Environmental Health, Research  
28 Unit Environmental Genomics, 85764 Neuherberg, Germany

29 <sup>11</sup>Department of Civil and Environmental Engineering, University of Cyprus, P.O. Box 20537,  
30 1678 Nicosia, Cyprus

31

32

33

34

35 **Running title:** Fate of antibiotics and related resistant elements in wastewater-irrigated  
36 agricultural systems

37

38

39

40

41 \*corresponding author: Tel:+357 22 403113, Fax: +357 22 316770, E-mail address:

42 [anastasis.christou@ari.gov.cy](mailto:anastasis.christou@ari.gov.cy) (A. Christou);

43 \*\*corresponding author: Tel:+357 22 893515, Fax: +357 22 895365, E-mail address:

44 [dfatta@ucy.ac.cy](mailto:dfatta@ucy.ac.cy) (D. Fatta-Kassinos)

45

46

47

## 48 *ABSTRACT*

49 The use of reclaimed wastewater (RWW) for the irrigation of crops may result in the continuous  
50 exposure of the agricultural environment to antibiotics, antibiotic resistant bacteria (ARB) and  
51 antibiotic resistance genes (ARGs). In recent years, certain evidence indicate that antibiotics and  
52 resistance genes may become disseminated in agricultural soils as a result of the amendment  
53 with manure and biosolids and irrigation with RWW. Antibiotic residues and other contaminants  
54 may undergo sorption/desorption and transformation processes (both biotic and abiotic), and  
55 have the potential to affect the soil microbiota. Antibiotics found in the soil pore water  
56 (bioavailable fraction) as a result of RWW irrigation may be taken up by crop plants,  
57 bioaccumulate within plant tissues and subsequently enter the food webs; potentially resulting in  
58 detrimental public health implications. It can be also hypothesized that ARGs can spread among  
59 soil and plant-associated bacteria, a fact that may have serious human health implications. The  
60 majority of studies dealing with these environmental and social challenges related with the use  
61 of RWW for irrigation were conducted under laboratory or using, somehow, controlled  
62 conditions. This critical review discusses the state of the art on the fate of antibiotics, ARB and  
63 ARGs in agricultural environment where RWW is applied for irrigation. The implications  
64 associated with the uptake of antibiotics by plants (uptake mechanisms) and the potential risks to  
65 public health are highlighted. Additionally, knowledge gaps as well as challenges and  
66 opportunities are addressed, with the aim of boosting future research towards an enhanced  
67 understanding of the fate and implications of these contaminants of emerging concern in the  
68 agricultural environment. These are key issues in a world where the increasing water scarcity  
69 and the continuous appeal of circular economy demand answers for a long-term safe use of  
70 RWW for irrigation.

71 **Keywords:** antibiotics; accumulation; human health risks; antibiotic-resistance genes; uptake;  
72 reclaimed wastewater irrigation

73

74 **Abbreviations:** APCI, atmospheric pressure chemical ionization; ARB, antibiotic resistant  
75 bacteria; ARGs, antibiotic resistance genes; CytCox, cytochrome c oxidase; DDA, data-  
76 dependent acquisition; DOM, dissolved organic matter;  $D_{ow}$ , pH-dependent speciation of ionic  
77 compounds; ESI, electrospray ionization; GSTs, glutathione S-transferases;  $H^+$ -ATPase, proton  
78 pump; HGT, horizontal gene transfer; HILIC, Hydrophilic interaction liquid chromatography;  
79  $K_{oc}$ , organic carbon-normalized sorption coefficient; LC HRMS, liquid chromatography high-  
80 resolution mass analyzers; LC, liquid chromatography; MAE, microwave-assisted extraction;  
81 MGEs, mobile genetic elements; NER, non-extractable residues; PCR, polymerase chain  
82 reaction; PLE, pressurized liquid extraction; QqQ, triple quadrupole; QqQ-LIT, hybrid triple  
83 quadrupole-linear ion trap; QuEChERS, quick, easy, cheap, effective, rugged and safe; ROS,  
84 reactive oxygen species; RWW, reclaimed wastewater; SLE, solid liquid extraction; SOM, soil  
85 organic matter; SRM, selected reaction monitoring; TPs, transformation products; TTC,  
86 threshold of toxicological concern; UHPLC, ultra-high performance liquid chromatography;  
87 USE, ultrasound-assisted solvent extraction; WWTPs, wastewater treatment plants;

88

89

90

91

92

93

94

95	
96	
97	<b>Contents</b>
98	1. Introduction
99	.....6
100	1.1. Why antibiotics and antibiotic resistance should be considered
101	as contaminants of emerging concern .....
102	.....6
103	2. Fate of antibiotics in reclaimed wastewater-irrigated agricultural soil
104	.....9
105	2.1. Sorption .....11
106	2.2. Transport in soil .....13
107	2.3. Transformations in soil .....15
108	3. Detection and quantification of antibiotics, ARB and ARGs in soils and crops.....18
109	3.1. Extraction and analysis of antibiotics in soils and crops
110	.....19
111	3.1.1. Extraction methodologies .....20
112	3.1.2. Instrumental analysis .....22
113	3.2. Detection and quantification of ARB and ARGs in RWW, soil and crop
114	samples.....25
115	4. Effects of antibiotics on soil biota (microbiome and soil fauna) .....26
116	5. Antibiotic resistant bacteria and resistance genes .....28
117	6. Uptake of antibiotics by reclaimed wastewater-irrigated crop plants in real and

118	simulated	field
119	conditions.....	33
120	7. Phytotoxic effects .....	
121	....	38
122	8. Public health implications / Risk assessment .....	39
123	9. Concluding remarks and recommendations for future research .....	41
124	References .....	45

125  
126  
127

## 128 1. Introduction

### 129 1.1. Why should antibiotics and antibiotic resistance be considered as contaminants of 130 emerging concern?

131 Since their introduction into medicine in the 1940s, antibiotics have been central to modern  
132 healthcare ([Center for Disease Dynamics and Economics & Policy, 2015](#); [Nesme and Simonet, 2015](#)). This has involved their extended usage for the treatment of serious infections related to  
133 human health and welfare and for the promotion of growth and disease prevention in livestock  
134 and other food animals. Together with population growth, increasing prosperity and  
135 inappropriate use, have stimulated the production of thousands of tons of antibiotics, with  
136 projections for further increased production in the forthcoming years ([Van Boeckel et al., 2014](#);  
137 [Van Boeckel et al., 2015](#)). Over the last decades, an increasing body of evidence has shown that  
138 antibiotics entering the environment, subsequently pose potential adverse effects on non-target  
139 organisms and humans ([Boxall, 2004](#); [Runnalls et al., 2010](#); [Vasquez et al., 2014](#); [Brandt et al., 2015](#)). Antibiotics are introduced into the environment via various human activities, including

142 direct disposal of unused or expired medication, release from pharmaceutical manufacturing  
143 plants and hospitals, and veterinary drug use (Grossberger et al., 2014). Moreover, most  
144 antibiotics are poorly absorbed and not completely metabolized in human and animal bodies.  
145 Hence, a high percentage of the intake dosage (30-90%) of most antibiotics is excreted via urine  
146 and faeces within hours after application either as the parent compound or as metabolites (Liu et  
147 al., 2010; Zhang et al., 2014). As a result, antibiotics may directly enter the environment through  
148 the application of manure to soil and excretion by grazing livestock (Pan and Chu, 2016a). The  
149 use of RWW for irrigation and the use of biosolids as soil amendments constitute additional  
150 significant pathways for the introduction of antibiotics in the agricultural environment, as  
151 conventional wastewater treatment processes are only moderately effective at removing  
152 antibiotics from the RWW (Michael et al., 2012; Petrie et al., 2015). It should be noted that the  
153 removal efficiency of antibiotics during wastewater treatment processes varies and is mainly  
154 dependent on a combination of antibiotics' physicochemical properties and the operating  
155 conditions of the treatment systems (reported concentration of antibiotics in RWW range from  
156 low  $\text{ng L}^{-1}$  to low  $\mu\text{g L}^{-1}$  depending on the type of antibiotic, the treatment technology applied in  
157 WWTPs and the season of the year) (Michael et al., 2012). The most commonly applied  
158 biological treatments (i.e. conventional activated sludge, membrane bioreactor, moving bed  
159 biofilm bioreactor) are usually unable to efficiently remove antibiotics from RWW (low removal  
160 efficiency especially for polar antibiotics); as a result the application of costly advanced  
161 treatment processes downstream of conventional biological process (i.e. membrane filtration  
162 such as reverse osmosis, activated carbon adsorption, activated oxidation processes such as  
163 ozonation, fenton oxidation or sonolysis) and disinfection (i.e. ultraviolet irradiation) should be  
164 applied for the significant improvement of antibiotics removal efficacy (up to 100% in many  
165 cases) (Michael et al., 2012; Luo et al., 2014). Consequently, antibiotics are routinely detected in



166 RWW and biosolids, and in RWW-irrigated agricultural soils and runoff from such sites,  
167 biosolids- and manure-amended soils, and surface and groundwater systems and sediments  
168 receiving RWW ([Kolpin et al., 2002](#); [Pedersen et al., 2003](#); [Kinney et al., 2006](#); [Fatta-Kassinos](#)  
169 [et al., 2011a](#); [Gottschall et al., 2012](#); [Luo et al., 2014](#); [Meffe and de Bustamante, 2014](#)). The  
170 reported uptake of antibiotics by crop plants and aquatic organisms and their subsequent entry  
171 into the human food web warrants special concern due to possible public health effects ([Rand-](#)  
172 [Weaver et al., 2013](#); [Malchi et al., 2014](#); [Pan et al., 2014](#); [Prosser and Sibley, 2015](#)).

173  
174 Along with antibiotics, RWW, biosolids and manure may carry significant loads of antibiotic  
175 resistant bacteria (ARB) and resistance genes (ARGs) ([Szczepanowski et al., 2009](#); [Munir and](#)  
176 [Xagorarakis, 2011](#); [Rizzo et al., 2013](#); [Manaia et al., 2016](#)). [Rizzo et al. \(2013\)](#) reviewed the  
177 spread of ARB and ARGs from WWTPs and concluded that conventional WWTPs are  
178 important hotspots for the spread and selection of ARB as well as ARG transfer, and that  
179 advanced treatment technologies (i.e. ozonation, fenton oxidation and sonolysis) and  
180 disinfection processes are regarded as possible tools to control the spread of ARB into the  
181 environment. While antibiotic residues may exert selective pressure on exogenous or on soil  
182 resident bacteria, the spread of ARB and ARG to terrestrial and aquatic and, eventually other  
183 environments, may be enhanced ([Bondarczuk et al., 2016](#)). WWTPs could be regarded as  
184 “genetic reactors” that assemble bacteria from a myriad of human and environmental sources  
185 and offer conditions that may favor the exchange of genetic material, ARB selection, and hence  
186 their rapid evolution ([Ju et al., 2016](#); [Manaia et al., 2016](#)). Simultaneously, the environmental  
187 matrices receiving RWW and biosolids constitute additional genetic reactors, where bacteria  
188 originated from the abovementioned sources may be mixed and counteract with environmental  
189 organisms ([Baquero et al., 2008](#)). Thus, the continuous disposal of RWW, biosolids and manure

190 in the environment contribute to the enrichment of soil with ARB and ARGs, with soil being  
191 already considered as one of the largest environmental reservoir of antibiotic resistance ([Nesme](#)  
192 [et al., 2014](#); [Nesme and Simonet, 2015](#)). ARGs may persist in the environment and, even worse,  
193 they can be transferred to other bacteria including human commensals or pathogens of clinical  
194 relevance, through horizontal gene transfer (HGT) of mobile genetic elements (MGEs). The  
195 implications of the widespread distribution of this type of contaminants can be substantial at  
196 both the public health and economic levels ([Han et al., 2016](#); [Johnson et al., 2016](#)). Reaching  
197 alarming levels in many parts of the world ([World Health Organization, 2014](#)), the increasing  
198 emergence and propagation of ARGs are threatening modern medicine and posing major risks to  
199 human health and ecological sustainability in the 21<sup>st</sup> century ([Bush et al., 2011](#); [Berendonk et](#)  
200 [al., 2015](#); [Price et al., 2015](#)).

201  
202 Diminishing availability of good quality freshwater due to the growing demand of an increasing  
203 world population and climate change-driven frequent and prolonged dry periods, render RWW  
204 as a valuable alternative water source in arid and semi-arid regions worldwide ([Bixio et al.,](#)  
205 [2006](#)). Currently, RWW is commonly used to irrigate agricultural land and urban greeneries, and  
206 replenish surface and groundwater resources ([Hamilton et al., 2007](#); [Pan et al., 2014](#)).

207  
208 This review collates recent knowledge on antibiotics and ARB and ARGs in the agricultural  
209 environment as a result of the use of RWW for irrigation. Among others, the effects of biotic  
210 and abiotic factors on the fate of these contaminants of emerging concern in the agricultural  
211 environment receiving RWW irrigation, as well as implications for plant uptake and potential  
212 negative effects on public health, will be discussed and highlighted.

213

214

## 215 **2. Fate of antibiotics in reclaimed wastewater-irrigated agricultural soil**

216 A great number and variety of antibiotics and related elements are present in RWW,  
217 constituting mixtures which may continuously vary (intra- and inter-daily, seasonally and inter-  
218 annually) in composition and concentrations ([Diwan et al., 2013](#); [Petrie et al., 2015](#)). Therefore,  
219 RWW irrigation may result in the continuous exposure of the agricultural environment to a  
220 variety of antibiotics and ARB and ARGs ([Wang et al., 2014](#)). RWW-irrigated soils have been  
221 found to accumulate antibiotics in concentrations that are several folds higher than the ones  
222 found in the irrigation water ([Kinney et al., 2006](#); [Calderón-Preciado et al., 2011](#)). [Kinney et al.](#)  
223 [\(2006\)](#) detected erythromycin in RWW-irrigated soils in Colorado State, USA, at concentrations  
224 of 0.02-15  $\mu\text{g kg}^{-1}$ . Furthermore, [Wang et al. \(2014\)](#) explored the effects of long-term RWW  
225 irrigation of six public parks in Beijing, China, on the concentration of five tetracyclines  
226 (tetracycline, oxytetracycline, chlortetracycline, methacycline, and doxycycline) and 9 of their  
227 degradation products, four sulfonamides (sulfadimethoxine, sulfamerazine, sulfamethizole,  
228 sulfamethoxazole), and six fluoroquinolones (ofloxacin, enrofloxacin, sarafloxacin,  
229 danofloxacin, ciprofloxacin, norfloxacin) in the rhizosphere soil. The total concentration of  
230 tetracyclines was in the range of 12.7-145.2  $\mu\text{g kg}^{-1}$ , with the parent compound being found in  
231 higher concentrations compared to their degradation products. Fluoroquinolones were randomly  
232 detected in sampled soils with their highest total concentration being 79.2  $\mu\text{g kg}^{-1}$ , whereas none  
233 of the four sulfonamides examined were found in all soil samples ([Wang et al., 2014](#)).  
234 [Grossberger et al. \(2014\)](#) found sulfamethoxazole in soil in concentrations ranging from 0.12-  
235 0.28  $\mu\text{g kg}^{-1}$  (depending on the soil type) following the irrigation of carrot crop with RWW for a  
236 single growing period.

237

238 Antibiotics are ionizable molecules and can occur as neutral and/or charged species  
239 (zwitterionic, negative or positive) in the RWW used for irrigation and in the receiving soil (Wu  
240 et al., 2015). The chemical form of antibiotics in soil pore water (bioavailable/bioaccessible  
241 fraction), along with the properties of soil and the surrounding water, shape the fate of  
242 antibiotics in agricultural soils. Once introduced into soil, antibiotics are subjected to  
243 sorption/desorption and transformation processes (both biotic and abiotic), which influence the  
244 concentrations available for biodegradation, transport into soil (runoff and leaching) and plant  
245 uptake, ultimately specifying the potential of accumulation of antibiotics in soil (Grossberger et  
246 al., 2014). The chemical properties of antibiotics that significantly impact and shape their  
247 environmental fate are polarity, hydrophobicity and water solubility. The polarity of organic  
248 compounds is determined by the presence of ionizable functional groups, such as carboxyl,  
249 phenolic hydroxyl and amine moieties within the molecules, which may be protonated and  
250 deprotonated depending on soil pore water pH, thus acquiring a positive or negative charge,  
251 respectively. As a result, polar and ionizable antibiotics engage in interactions with the soil  
252 organic matter (SOM), the mineral surfaces and the dissolved organic matter (DOM). Such  
253 interactions include hydrophobic partitioning, electron donor-acceptor interactions (e.g.,  
254 hydrogen bonding), cation-anion exchange, protonation, water binding, cation binding and  
255 surface complexation (Thiele-Bruhn et al., 2004; Vasudevan et al., 2009). Therefore, the  
256 physicochemical properties of antibiotics as well as the chemistry of soil pore-water (i.e. pH,  
257 mineral concentration, cation exchange capacity, dissolved organic matter), and the soil organic  
258 matter (SOM) content and structure (i.e. clay composition) are critical factors controlling the  
259 retention of antibiotics in soil (Vasudevan et al., 2009; Wu et al., 2013a; Miller et al., 2016; Park  
260 and Huwe, 2016) (Table 1).

261

262 In the following sections, studies concerning the main processes affecting the environmental fate  
263 of antibiotics in soils receiving RWW for irrigation (i.e. sorption, transformation and transport)  
264 are presented. These studies are the ones performed under real-scale applications or under  
265 controlled condition simulating RWW irrigation. Other controlled studies (performed either in  
266 the lab or in greenhouse) that corroborate the limited findings regarding the environmental fate  
267 of antibiotics in the agricultural environment receiving RWW for irrigation, are also discussed.

268

## 269 **2.1. Sorption**

270 Polar and ionizable antibiotics tend to remain soluble in soil pore water rather than be retained in  
271 soil organic matter and mineral surfaces, whereas, conversely, nonpolar and moderately polar  
272 neutral antibiotic compounds are instantaneously absorbed by SOM, while also spontaneously  
273 get desorbed to soil water till an equilibrium is established ([Thiele-Bruhn et al., 2004](#); [Wegst-  
274 Uhrich et al., 2014](#))([Table 1](#)). Partitioning of antibiotics between SOM and soil pore water is  
275 typically described using an organic carbon-normalized sorption coefficient,  $K_{oc}$  (compounds  
276 with  $\log K_{oc}$  values  $<2$  are considered to be capable for only weak sorption) but equilibrium  
277 partitioning isotherms in many cases do not fit with linear models ([Chefetz et al., 2008](#); [Xu et  
278 al., 2009](#); [Revitt et al., 2015](#); [Park and Huwe, 2016](#)). To this effect, the Freundlich isotherms has  
279 been revealed to be the most successful ones to describe the sorption of antibiotics to soil  
280 ([Chefetz et al., 2008](#); [Xu et al., 2009](#); [Revitt et al., 2015](#); [Park and Huwe, 2016](#)) Interestingly,  
281 [Tulp et al. \(2008\)](#) reported a reduction of polar, multifunctional compounds partitioning  
282 behaviour over a large range of environmental matrices by a factor of 7-60, compared with  
283 nonpolar and moderately polar neutral compounds. Currently, the ability to predict sorption for  
284 ionized organic compounds to SOM from solute descriptors is limited, although sorption of  
285 organic anions to SOM is generally lower than that of the corresponding neutral species ([Miller](#)

et al., 2016). Moreover, the extent of association of polar ionizable antibiotics with soil particles and SOM is strongly determined by the chemistry of soil pore water (i.e. pH, ionic strength, concentration of competing ions) (Gu et al., 2007; Vasudevan et al., 2009; Kodešová et al., 2015). Kurwadkar et al. (2007) have observed that sulfathiazole and sulfamethazine demonstrated a strong pH dependency for sorption in three soils having distinguished texture (i.e. loamy sand, sandy loam and loam), showing higher sorption capacity at soil pore water pH values lower than 7.5, where these antibiotics exist primarily in their neutral/cationic form. Weakly acidic antibiotics (i.e. sulfamethoxazole), which regularly carry a negative charge in RWW-irrigated soils, may be poorly retained by soil particles and SOM due to repulsion forces between the deprotonated radicals and the negatively charged soil particles and SOM, consequently being prompted to leaching; thus are regularly being detected in aquifers (Chefetz et al., 2008; Borgman and Chefetz, 2013). Moreover, compounds that display higher hydrophobicity are adsorbed to a lower extent in organic soils with high clay content (Durán-Álvarez and Jiménez-Cisneros, 2014), showing significant rate of desorption and higher potential for reaching the aquifer during rainfall events or continuous RWW irrigation (Chefetz et al., 2008). Conversely, positively charged antibiotics, such as tetracyclines, may be retained onto soil particles by cation exchange, while simultaneously desorption at low rates may occur as a result of competition with metal and organic cations (Gu et al., 2007). Antibiotics that carry both positive and negative charge due to the existence of several functional groups in their complex structure (i.e. ciprofloxacin) can undergo both sorption and desorption from soil mineral surfaces and soil organic matter (Wu et al., 2013a). Importantly, these processes are strongly pH-dependent and vary in the presence of metal cations, probably due to surface complexation with  $Al^{3+}$ ,  $Na^{+}$  and  $Ca^{2+}$  (Vasudevan et al., 2009; Wu et al., 2013a). Overall, the soil pore water pH can be considered as a dominant factor controlling the sorption of polar and

310 ionizable antibiotics in soil. Ionizable antibiotics regularly show lower sorption rates in alkaline  
311 soils, whereas lower rates of sorption may be registered in acidic soils receiving RWW  
312 irrigation, as RWW regularly has pH values greater than 7 (7 to 8), which may result in elevated  
313 pH values of the soil pore water ([Borgman and Chefetz, 2013](#); [Christou et al., 2017](#)).

314

315 The effects of rhizosphere in antibiotics speciation and bioavailability should also be taken into  
316 account, as the root exudates, which may contain organic acids such as citric, oxalic and malonic  
317 acids, can reduce the soil pore water pH up to two units as far as 2-3 mm from the root surface  
318 compared with bulk soil ([Hinsinger et al., 2003](#)). Such alteration in the rhizosphere pH values  
319 may influence the properties of soil, the microbial mineralization of SOM ([Keiluweit et al.,](#)  
320 [2015](#)), and therefore the speciation and sorption of neutral and ionizable antibiotics ([Hurtado et](#)  
321 [al., 2016](#)).

322

## 323 **2.2. Transport in soil**

324 Antibiotics may be transported to soil if dissolved in soil pore water, either vertically leading to  
325 their presence in deeper soil depths and the aquifer, or horizontally, causing contamination of  
326 unpolluted sites and adjacent water bodies ([Alder et al., 2001](#); [Davis et al., 2006](#)). The migration  
327 of antibiotics in soil is closely related to their sorption capacity onto the soil matrix, influenced  
328 by their physicochemical properties, the properties of soil and the chemistry of soil pore water  
329 ([Chefetz et al., 2008](#); [Zhang et al., 2014](#); [Park and Huwe, 2016](#)) ([Table 1](#)). Transport studies can  
330 be performed using different approaches in the laboratory, either by using packed soil columns  
331 or undisturbed soil columns tests. The presence of expansive clays in soil results in the  
332 disappearance of preferential path in the porous network of soil once clay becomes wet, which in  
333 turn provokes the decay in transport of organic contaminants contained in soil pore water

334 ([Durán-Álvarez and Jiménez-Cisneros, 2014](#)). Moreover, increases in SOM, due to manure,  
335 compost or biosolids soil amendment, may also result in decreased mobility of antibiotics in  
336 RWW-irrigated soils as a result of enhanced sorption ([Borgman and Chefetz, 2013](#)). In addition,  
337 DOM inputs due to RWW irrigation can affect transport and sorption of antibiotics in  
338 agricultural soils. DOM can increase the antibiotics' apparent solubility and therefore enhance  
339 their mobility (facilitated by co-transport), or conversely, reduce their mobility due to co-  
340 sorption and cumulative sorption to the soil's solid phases ([Chefetz et al., 2008](#); [Haham et al.,](#)  
341 [2012](#)) ([Table 1](#)). These contradictory effects may be attributed to the fact that the processes  
342 affecting the mobility of antibiotics and DOM, as well as of their complex, are controlled by the  
343 binding affinity of antibiotics to the DOM, the DOM-antibiotics complexation kinetics as well  
344 as by the hydrophobicity of DOM and the DOM-antibiotics complex, which in turn determine  
345 their binding affinity to the soil organic and inorganic matrices ([Chefetz et al., 2008](#)). Therefore,  
346 the mobility of the antibiotics may be increased or decreased if the binding affinity of the DOM  
347 and DOM-antibiotics complex to the soil matrices is low or high, respectively.

348  
349 [Kinney et al. \(2006\)](#) reported that the differences in concentrations of erythromycin and  
350 sulfamethoxazole in 5-cm increments of a soil profile (0-30 cm soil layer) in RWW-irrigated  
351 fields might indicate interactions of these antibiotics with soil components during leaching  
352 through the vadose zone. Indicatively, sulfamethoxazole has been detected at a mean  
353 concentration of  $0.11 \mu\text{g L}^{-1}$  in groundwater beneath soils subjected to long-term irrigation (45  
354 years) with RWW (secondary treated, activated sludge) in Germany ([Ternes et al., 2007](#)). The  
355 application of biosolids was found to increase the retardation of antibiotics in soils, thus  
356 mitigating their leaching potential, whereas RWW irrigation may increase the mobility of  
357 weakly acidic antibiotics, as the elevation of soil pore water pH due to the neutral-basic nature



of RWW results in the predominant presence of the anionic form of these antibiotics in soil (the repulsion of antibiotics from the negatively charged soil surfaces enhance the leaching potential) (Borgman and Chefetz 2013). Bondarenko et al. (2012) evaluated the ability of turfgrass systems in attenuating trimethoprim and sulfamethoxazole antibiotics during RWW irrigation by monitoring the leachate water at the 90-cm depth, and revealed the higher leaching potential of trimethoprim compared with sulfamethoxazole, as the former displayed higher concentrations and frequency of detection in the leachate over the latter. Horizontal transport of antibiotics may result from runoff, or the deposition of RWW aerosol close to irrigation channels, the deposition of soil material derived from RWW-irrigated sites by wind erosion, or the transport of soil material between fields with farm machinery (Dalkmann et al., 2012).

368

### 2.3. Transformation in soil

The bioavailable/bioaccessible concentrations of antibiotics in wastewater-irrigated soils may be altered by abiotic and biotic (microbial) transformation processes. Such processes may result in the mineralization of these organic molecules, or the formation of biologically active transformation products that may be taken up by plants (Jechalke et al., 2014; Miller et al., 2016). Abiotic transformation processes that may take place in agricultural soils include photolysis, hydrolysis and redox reactions (Table 1). Although these transformation processes of antibiotics are well documented in wastewater treatment plants and receiving water sources (Fatta-Kassinos et al., 2011b; Homem and Santos, 2011; Ganiyu et al., 2015), only limited information is available regarding the abiotic transformation processes of antibiotics occurring in soil. Direct photolysis of antibiotics in soils is considered trivial due to light attenuation (Hebert and Miller, 1990), since the SOM and DOM can act as quenchers of UV irradiation decreasing the photodegradation kinetics of antibiotics compared with clean water (Fatta-

382 [Kassinis et al., 2011b](#)). While no evidence exists regarding the hydrolysis of antibiotics in  
383 RWW-irrigated soils,  $\beta$ -lactam antibiotics were found to be rapidly hydrolyzed in manure-  
384 amended soils ([Jechalke et al., 2014](#)). In addition, [Sollicec et al. \(2016\)](#) found tetracycline  
385 antibiotics in the range of  $\mu\text{g kg}^{-1}$  in soils following swine manure amendment, while  
386 degradation products resulting from oxidation, hydrolysis and biodegradation processes were  
387 sometimes found at higher concentrations compared with the ones of the parent compounds.

388

389 Microbial biodegradation and biotransformation are considered to be dominant biotic processes  
390 that greatly shape the fate of antibiotics in soil ([Lin and Gan, 2011](#); [Ding et al., 2016](#); [Pan and](#)  
391 [Chu, 2016a](#)). The biotic transformation of antibiotics in soil was found to be influenced by their  
392 initial concentrations, microbial activities, oxygen status in the soil, soil type and environment  
393 (moisture, temperature, salinity, pH), the presence of SOM and clay content, and the  
394 physicochemical properties of the antibiotic ([Table 1](#)) ([Lin and Gan, 2011](#); [Grossberger et al.,](#)  
395 [2014](#); [Pan and Chu, 2016a](#)). The biodegradation of antibiotics in soil is also influenced by their  
396 sorption capacity to soil matrices, which in turn determines their bioavailable fraction in soil  
397 pore water. Therefore, soil characteristics such as the SOM content, soil texture and soil pH  
398 greatly shape the rates of antibiotics degradation in RWW-irrigated soils ([Lin and Gan, 2011](#);  
399 [Wu et al., 2012](#); [Durán-Álvarez and Jiménez-Cisneros, 2014](#)). High SOM and clay content often  
400 correlate with decreased biodegradation rates, probably due to the reduced bioavailability of  
401 antibiotics because of the increased sorption to SOM (mainly humic acids) ([Xu et al., 2009](#); [Wu](#)  
402 [et al., 2012](#)) and the formation of non-extractable residues (NER) ([Yang et al., 2009](#); [Müller et](#)  
403 [al., 2013](#)). Biosolids or manure amendment may either reduce biodegradation due to increase  
404 sorption or increase biodegradation due to enhanced microbial activity ([Walters et al., 2010](#)).  
405 Generally, the biodegradation of antibiotics in soil is faster and more complete under aerobic

(topsoil) as compared to anaerobic conditions (deeper layers of vadose zone) (Liu et al., 2010; Wu et al., 2012; Pan and Chu, 2016a). However, it is widely accepted that both aerobic and anaerobic processes are needed for the overall biodegradation of antibiotics in RWW-irrigated soils. Aerobic biodegradation may result in the fast initial enzymatic attack and the decomposition of alkyl side-chains and other easily degradable functional groups (e.g. carboxyl groups), whereas anaerobic biodegradation may include various enzymatic processes that biodegrade more complex and stable functional groups and structural moieties, such as aromatic groups, naphthalene rings and sulfonamides (Ghattas et al., 2017). Low concentration of antibiotics in soils (in the range of low ng kg<sup>-1</sup>) in fields irrigated with RWW for the short time may result in limited biodegradation rates, indicating that higher concentrations (i.e. due to prolonged RWW irrigation or irrigation with RWW containing high concentrations of antibiotics) are necessary to induce changes in the soil microbial community structure or to promote the expression of biodegradation enzymes (Grossberger et al., 2014; Miller et al., 2016). Moreover, the degradation rates of macrolide antibiotics were found to be accelerated in soils that were previously exposed to antibiotics as a result of RWW irrigation or biosolids amendment (Topp et al., 2016). Nevertheless, Grossberger et al. (2014) reported contradictory results, as pre-exposure of soils to sulfamethoxazole via RWW irrigation did not enhance its biodegradation rate. Wang et al. (2014) reported the presence of nine biodegradation products of tetracycline antibiotics in the rhizosphere soil of RWW-irrigated parks in Beijing, China, with 4-epianhydrochlortetracycline being detected in all soil samples in the range of 1.3-5.1 µg kg<sup>-1</sup>. Biotic transformation of antibiotics in the rhizosphere may be increased compared with that in bulk soil (Kopmann et al., 2013), as plants and rhizosphere-associated microorganisms secrete enzymes, such as laccases and peroxidases, that can effectively biodegrade antibiotics; laccase oxidation was reported as an efficient mechanism for the removal of sulfonamide antibiotics

430 from soil ([Ding et al., 2016](#)). The limited information with regard to the biotransformation of  
431 antibiotics in soils irrigated with RWW is due to the small number of studies conducted under  
432 real field conditions. Therefore, there is a need for further studies, since the transformation  
433 products (TPs) may exert biological effects or be taken up by crop plants.

434

435

### 436 **3. Detection and quantification of antibiotics, ARB and ARGs in soils and crops**

437 The detection and quantification of antibiotics and/or ARB and ARGs in soil and crop samples  
438 are laborious and challenging tasks, although possible nowadays, thanks to developments in  
439 analytical instrumentation and techniques. The wide range of extraction protocols and the  
440 chromatographic techniques, along with the challenges raised, for the detection and  
441 quantification of antibiotics are discussed in detail. Cultivation based methods for the  
442 characterization of antibiotic resistance, and molecular biology methods applied for revealing the  
443 diversity and the abundance of ARB and ARG in RWW and in soil samples have been reviewed  
444 by different authors ([Rizzo et al., 2013](#); [Luby et al., 2016](#); [Manaia et al., 2016](#)) and hence are  
445 summarized in [Fig. 2](#).

446

#### 447 **3.1. Extraction and analysis of antibiotics in soils and crops**

448 The determination of antibiotics in the agricultural environment including soils and crops is  
449 necessary for getting a better understanding of their fate, impact and human exposure  
450 assessment. This objective has been achieved only lately, attributed to the developments in  
451 analytical instrumentation and techniques that have enabled researchers to detect and quantify  
452 such organic micro-pollutants in these environmental matrices. However, because of the  
453 relatively recent interest in such studies, there is still a lack of validated and standardized

454 protocols of analysis that guarantee the quality and fit to purpose of the results obtained. The  
455 main difficulties in the analysis of antibiotics in agricultural soils and crops are associated to the  
456 low concentration expected of these compounds, especially in the vegetal material, and the  
457 complexity of these matrices, which contain large amounts of endogenous components, like  
458 organic matter, pigments and fatty or waxy materials, which can interfere in the determination.

459

460 To evaluate the uptake and distribution of antibiotics in plants, different parts of crops, plant  
461 leaves, stems, roots and fruits, must be analyzed separately. If we add to this the large variety of  
462 soils and crops that can be studied, we can get an idea of the complexity of the analytical  
463 problem, since validated methods must be developed for specific matrices presenting differences  
464 in matrix effects. Other difficulties arise from differences in the structure and physicochemical  
465 properties of the antibiotics, which not only affect the behavior of these compounds in the  
466 agricultural environment as it is discussed in this paper, but also the extraction efficiency and  
467 analysis. For example, the strong interaction of tetracyclines with organic matter and clay  
468 components in soils results in their poor extraction, as well as in lowering the reproducibility of  
469 the measurements ([Kulshrestha et al., 2004](#)), while the pH values greatly influence the extraction  
470 of tetracyclines and fluoroquinolones from soil and vegetables ([Hu et al., 2014](#)). Consequently,  
471 reported methods usually include a limited number of target analytes, and a method for multi-  
472 residue determination of a broad range of antibiotics in soils and plants at environmentally  
473 relevant levels is demanded.

474

475 Finally, many antibiotics are metabolized or degraded after use, resulting in the formation of  
476 transformation products (TPs), which can be present in the irrigation water or can be generated  
477 once the antibiotics reach the agricultural media. These TPs represent a risk still not evaluated

478 since scarce information about type and behavior of TPs present in the soil-plant system is  
479 available and effort has to be paid in developing analytical strategies for their identification.

480

### 481 **3.1.1. Extraction methodologies**

482 Previous to the analysis, a sampling strategy must be carefully designed in order to obtain  
483 representative samples, which provide reliable results. The type of soil and crop and agricultural  
484 practices in real conditions are crucial in defining the sampling protocol. Once the sample is  
485 obtained, sample preparation, including pretreatment and extraction, is critical to assure  
486 maximum recovery together with efficient removal of potential interferences. Pretreatment is  
487 usually focused to sample homogenization, and includes cutting, grinding, blending, sieving and  
488 lyophilization steps. As a common practice, plant materials are rinsed with deionized water to  
489 discriminate between contaminant deposition and uptake, whereas soils are dried at room  
490 temperature or lyophilized, before extraction ([Matamoros et al., 2012](#)).

491

492 With regard to the extraction, several techniques have been tested ([Agüera and Lambropoulou,](#)  
493 [2016](#)). Those more commonly reported include solid liquid extraction (SLE) ([Hawker et al.,](#)  
494 [2013](#)), ultrasound-assisted solvent extraction (USE) ([Sollicet et al., 2016](#); [Koba et al., 2017](#)),  
495 pressurized liquid extraction (PLE) ([Jacobsen et al., 2004](#); [Franklin et al., 2016](#); [Azanu, et al.,](#)  
496 [2016](#)) or more recently QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extraction  
497 ([Hu et al., 2014](#); [Salvia et al. 2012](#)). [Table 2](#) includes, as an example, information of some of the  
498 reported methods. In most of the cases water, methanol, acetone, ethyl acetate and acetonitrile  
499 are the solvents of choice, in combination with buffer solutions (McIlvaine buffer, citrate buffer)  
500 and chelating agents (EDTA) that are being used to avoid the formation of chelate complexes  
501 between some antibiotics (tetracyclines) and metal ions present in soils ([Li et al., 2011a](#)). PLE

502 and USE have been extensively used to extract various classes of antibiotics from soil (Jacobsen  
503 et al., 2004; O'Connor and Aga, 2007) and recently also from plant material, though a post-  
504 extraction “cleanup” is often required to remove co-extracts. SPE is the most widely used clean-  
505 up procedure and thus the extract is directly percolated or diluted in water (<5% organic content)  
506 and then percolated through the cartridge. Polymeric materials are the sorbents of choice because  
507 of its ability to retain compounds in a wide range of polarities.

508

509 Recently, the well-known QuEChERS extraction method, extensively applied in pesticide multi-  
510 residue analysis in crops, has extended its application to other environmentally relevant analytes  
511 and matrices (Bruzzoniti et al., 2014). In most of the cases, an appropriate method optimization  
512 is required and modified versions of the original procedure have been reported. Hu et al. (2014)  
513 propose the application of a modified QuEChERS method for determination of 26 veterinary  
514 antibiotics in vegetables. In this case, acetonitrile:methanol (85:15, v/v) was selected as the  
515 extraction solvent and the buffer composition was modified to increase the acidity of the  
516 extraction system in order to improve fluoroquinolones and tetracycline extraction. Recoveries  
517 higher than 60% were obtained for 23 of the 26 antibiotics tested. The use of UAE (Ferhi et al.,  
518 2016) and changes in the clean-up step (Salvia et al., 2012) have been also introduced for  
519 improving the recovery of antibiotics in soils.

520

### 521 3.1.2. Instrumental analysis

522 At present, liquid chromatography (LC) in combination with mass spectrometry (MS) is the  
523 primary technology used to analyze multiple antibiotics in wastewater as well as in soil and plant  
524 tissue extracts. Innovation in the theory and development of novel chromatographic columns has  
525 made reversed phase (RP) LC an accurate and reliable method for the determination of antibiotic

526 residues (Seifrtová et al., 2009). The extra resolution provided by Ultra-High Performance  
527 Liquid Chromatography (UHPLC) systems gives greater information and reduces the risk of not  
528 detecting potentially important co-eluting analytes. In the last years, the use of sub-2  $\mu\text{m}$ , or sub-  
529 2  $\mu\text{m}$  core-shell columns steadily increased (Salvia et al., 2012; Sollicec et al., 2016). Typically,  
530  $\text{C}_{18}$ , modified or not with more polar functional groups (Hawker et al., 2013; Huang et al., 2015;  
531 Franklin et al., 2016) is by far the most widely used stationary phase; However, other phases (i.e  
532 phenyl,  $\text{C}_8$  etc.) that offer different retention characteristics are also used (Hu et al., 2014; Azanu  
533 et al., 2016). As regards the mobile phase, in multi-residue studies, gradient elution of mixtures  
534 of water/acetonitrile or water/methanol is regularly reported. Volatile additives (e.g. formic acid,  
535 acetic acid, and ammonium acetate) at different concentration are used to modify mobile phase in  
536 order to improve the ionization of analytes and the MS detection sensitivity in the analysis of  
537 antibiotics, as well as to control pH (Hawker et al., 2013; Azanu et al., 2016).

538

539 With respect to mass spectrometers, tandem MS involving atmospheric pressure ionization, is  
540 nowadays state of the art, since these kind of analyzers combine two or more mass-to-charge  
541 ratio separation devices of the same or different types. Triple quadrupole (QqQ) and hybrid triple  
542 quadrupole-linear ion trap (QqQ-LIT) are among the most common and well established  
543 analyzers for target multi-residue methods (Azanu et al., 2016; Pan and Chu, 2016a). Selected  
544 Reaction Monitoring (SRM) is the preferred choice as acquisition mode. In this mode, selection  
545 of at least two specific transitions (precursor ion/product ion) is needed to fulfill requirements for  
546 a reliable quantification and confirmation of the analytes in the sample. Electrospray ionization  
547 (ESI), either positive (PI) or negative (NI), is usually the ionization mode of choice as it is more  
548 efficient for polar and ionizable compounds. In general, acidic groups are more compatible with  
549 NI whereas the presence of amine groups provides better performance in PI. Nevertheless, if



550 wide scope methods are designed, this behavior is hardly predictable when multiple groups are  
551 present and both modes should be used. In multi-analyte methods, polarity-switching during the  
552 same run (available in modern instruments) between PI and NI may be necessary to cover the  
553 range of compound classes ([Seifrtová et al., 2009](#); [Farouk et al., 2015](#)). Both, PI and NI modes  
554 were evaluated for the analysis of different classes of antibiotics (tetracyclines, macrolides,  
555 sulfonamides). Tetracyclines and macrolide antibiotics can be easily protonated and analyzed in  
556 PI mode. Sulfonamides can be readily detected in both NI and PI modes.

557

558 Signal suppression from the sample matrix and isobaric spectral interferences are among the  
559 major drawbacks of ESI mode that affect the sensitivity and should be always taken into  
560 consideration ([Sollicec et al., 2016](#); [Koba et al., 2017](#)). For this reason, the atmospheric pressure  
561 chemical ionization (APCI) mode was proposed as an alternative approach ([Schlüsener et al.,](#)  
562 [2003](#)). To overcome some of the ESI difficulties related to the matrix effects, matrix-matched  
563 calibration methodology is also applied ([Koba et al., 2017](#)).

564

565 Despite the tremendous technological advances of tandem triple quadrupole mass spectrometry  
566 in antibiotic target analysis, its use in non-target screening is still challenging. The desirable goal  
567 is the development of quantitative multiclass methods fully replacing the screening/confirmation  
568 traditional scheme, by expanding the ability to monitor a wider range of analytes, at the same  
569 time. In this context, the gradual introduction of high-resolution mass analyzers (LC HRMS),  
570 such as time-of-flight (TOF), Orbitrap, and hybrid mass spectrometer of quadrupole-time of-  
571 flight (Q-TOF) or Q-Orbitrap), has changed the mass spectrometry landscape for the  
572 determination of antibiotic residues, with non-target analysis. Among the different HRMS  
573 instruments, TOF is the least applied probably because it cannot operate as tandem MS

574 spectrometer. On the other hand, hybrid QqTOF and Orbitrap with a linear ion trap (LTQ-  
575 Orbitrap) or a quadrupole (Q-Orbitrap) are recently applied for target and non-target screening  
576 (Laganà and Cavaliere, 2015; Senyuva et al., 2015; Tong et al., 2016). More specifically, in a  
577 very recent study, LC-Q-Orbitrap MS was applied for selective quantification and identification  
578 of veterinary antibiotics and their various transformation products in soils (Sollicec et al., 2016).  
579 The use of hybrid quadrupole Orbitrap operated in a combination of full scan and fragmentation  
580 events named data-dependent acquisition (DDA) has previously been employed for the research  
581 of unknowns. The proposed extraction procedure (liquid solid extraction and solid-phase  
582 extraction were used for sample pre-concentration and purification) and the detection technique  
583 showed to be adequate, with LOD values ranging from 1.0 to 7.4  $\mu\text{g kg}^{-1}$  for soils. Similarly,  
584 Koba et al. (2017), demonstrated the successful use of LC-Q-Orbitrap MS for the accurate  
585 determination of three antibiotics (clindamycin, sulfamethoxazole, and trimethoprim) and five of  
586 their metabolites in different soils matrices.

587

588 Overall, most of the published methods for the measurement of antibiotics in soil and plant  
589 matrices are designed to analyze several compounds belonging to the same family. Methods  
590 covering several families of antibiotics are still scarce. The current strategy is focused towards  
591 multi-residue and multi-class methods for the simultaneous determination of antibiotic  
592 compounds having different physicochemical properties. To this effect, HRMS and especially  
593 orbitrap-HRMS is the promising technology within this area and is expected to be also the clear  
594 leader in the antibiotic crop uptake studies.

595

596 **3.2. Detection and quantification of ARB and ARGs in RWW, soil and crop samples**

597 The occurrence and abundance of ARB and ARG in RWW and soil samples may be determined  
598 either based on culture-dependent methods or on the direct analyses of total DNA (see Fig. 2).  
599 Wastewater samples need to be concentrated prior to analyses and hence the membrane filtration  
600 method is frequently used, either for bacterial cultivation or total DNA extraction. In contrast,  
601 soil samples may be suspended in water to desorb bacteria prior to cultivation, or be used  
602 directly for DNA extraction (Fig. 2). Given the fact that culture-dependent methods are more  
603 laborious, time-consuming and generally regarded as less informative, than culture-independent  
604 approaches, they have been successively replaced by metagenomics and quantitative PCR  
605 methods (Szczepanowski et al., 2009; Nesme et al., 2014). However, culture dependent  
606 methods, in particular targeting enterococci and *Escherichia coli*, are still relevant approaches to  
607 target indicator bacteria. These methods, besides allowing a direct comparison with routine  
608 monitoring analyses, if adequately adapted for instance with the supplementation of antibiotics,  
609 may lead to the detection of rare resistance phenotypes and genotypes (da Costa et al., 2006;  
610 Varela et al., 2013; Varela et al., 2015). Bacterial isolates may be characterized for the antibiotic  
611 resistance profiles and the mobile genetic elements or ARGs, supporting a comprehensive survey  
612 of the multidrug resistance phenotypes or capability to spread resistance by HGT. Culture  
613 independent methods are nowadays simpler to perform and have the important advantage of  
614 allowing the detection of ARGs in non-culturable bacteria. These approaches can target specific  
615 ARGs or related genes, and are being used as quantitative methods (quantitative PCR, qPCR) or  
616 as qualitative analyses (metagenomics). The method of qPCR, if adequately calibrated, allows  
617 the measurement of the abundance of a specific gene per volume of wastewater or gram of soil  
618 (and plant tissue as well), or of the prevalence of that same gene, expressed as the ratio between  
619 the gene under analysis and a housekeeping gene, normally 16S rRNA. In metagenomics  
620 analyses the relative abundance of a given gene is normally expressed as a ratio between the

number of sequence reads of the analysed gene vs the total number the reads or in relation to the number of reads of the 16S rRNA gene. Next generation sequencing analyses offer the possibility to have a broad view of ARGs, MGEs and bacterial populations, allowing simultaneously a good insight of the bacterial phylogenetic diversity in RWW or soil (Nesme et al., 2014). Not much is known about the occurrence of endophytic ARB in crops and this is in part due to technical challenges, referring mainly to low abundance of bacteria in such habitats.

#### **4. Effects of antibiotics on soil biota (microbiome and soil fauna)**

Antibiotics are regarded as persistent or ‘pseudo-persistent’ environmental contaminants of emerging concern. The ecotoxicological effects of antibiotics have been extensively studied, mainly with regard to the aquatic environment and the analysis of microorganisms; however, evidence on ecotoxicological effects on soil biota is still scant (Puckowski et al., 2016). Due to their persistence and known mode of action, antibiotics entering the soil are likely to disturb the complex regulatory networks in the soil microbiome and soil fauna, which are closely associated with soil quality and ecological function (Wardle et al., 2004; Becerra-Castro et al., 2015; Lopes et al., 2015). The effects of antibiotics on soil biota depend essentially on their bioavailability and, therefore, on soil properties, as well as on the availability of nutrients and the presence of root exudates (Halling-Sørensen et al., 2003; Bernier et al., 2011). Assays targeting the impact of antibiotics on soil microbial community function include microbial growth, respiration and enzyme activity, as well as functional diversity based on the community-level physiological profiling approach (culture independent methods) (Becerra-Castro et al., 2015; Brandt et al., 2015). The effects of antibiotics on soil bacteria, due to the application of antibiotic-contaminated manure or the artificial contamination of soil, are well documented using

645 controlled pot experiments (Reichel et al.; Schmitt et al., 2005; Liu et al., 2009; Schauss et al.,  
646 2009; Lin et al., 2016). For example, Baguer et al. (2000) reported that tylosin and  
647 oxytetracycline antibiotics spiked in soil at environmentally relevant concentrations had no  
648 effect on earthworms, springtails and enchytraeids, following 21 days of incubation (the lowest  
649 observed effect concentration was 3000 mg kg<sup>-1</sup>). Yu et al. (2011) showed that the observed  
650 behavior and growth defects (body bending frequency, reversal movement, omega turns and  
651 body length) of nematodes (*Caenorhabditis elegans*) following their exposure to  
652 sulfamethoxazole at environmentally relevant concentrations (1 ng L<sup>-1</sup> - 100 mg L<sup>-1</sup>) for 96 h  
653 transferred to the progeny. In addition, by using molecular assays, Pike and Kingcombe (2009)  
654 showed that the bacterial endosymbiont *Wolbachia*, which causes a variety of reproductive  
655 peculiarities, were successfully eliminated from the diplodiploid collembolan *Folsomia candida*  
656 through the continuous exposure of the populations (over two generations and several weeks) to  
657 rifampicin administered as 2.7% dry weight of their yeast food source, leading to the total  
658 sterility of all individuals of *Folsomia candida*, despite the continuation of normal egg  
659 production. Collectively, direct evidence indicating the impact of antibiotics on the microbial  
660 function and community structure in the agricultural environment as a result of RWW irrigation  
661 is extremely scarce. More precisely, only Ma et al. (2016) verified the effects of antibiotics  
662 applied through irrigation on soils microbial community, by conducting a controlled pot  
663 experiment using topsoil (0-20 cm) from a vegetable field following long-term RWW irrigation  
664 in northeast China. The studied soil was sprayed daily (for 120 days) with aqueous  
665 oxytetracycline solution in order to add 0.03 mg kg<sup>-1</sup> day<sup>-1</sup> of the antibiotic, aiming to mimic  
666 concentrations relevant in RWW irrigation. The daily oxytetracycline treatments promoted  
667 microbial carbon biomass (up to 2.64 times) and increased the McIntosh index of diversity  
668 ( $p < 0.05$ ) between 60 and 90 days as calculated using Biolog data and compared with the zero

oxytetracycline control, indicating a slight recovery in the soil microbial community and function (Ma et al., 2016).

Overall, RWW irrigation may introduce multiple changes to the soil biota, though it is extremely difficult to attribute such effects solely to antibiotics, as irrigation itself, or the introduction of other contaminants of emerging concern, or DOM or nutrients through RWW, may be responsible for such changes. Therefore, the effects of antibiotics on soil fauna due to RWW can be verified only through the performance of controlled experiments, where antibiotics may be applied either individually (see Ma et al., 2016) or in mixtures.

## **5. Antibiotic resistant bacteria and resistance genes**

Irrigation with RWW may result in the continuous release of ARB and ARGs to natural and agricultural environments (Fatta-Kassinos et al., 2011a; Negreanu et al., 2012; Gatica and Cytryn, 2013; Rizzo et al., 2013), which in turn can potentially cause risks to human health, as human-associated susceptible pathogenic bacteria can become resistant by acquiring resistance genes or other organism that are already resistant in the soil environment (Berendonk et al., 2015). In the last years, a plethora of studies revealed the presence of ARB and ARGs in the RWW of WWTPs worldwide (da Costa et al., 2006; Szczepanowski et al., 2009; Munir et al., 2011; Gao et al., 2012). Advanced wastewater treatment processes (i.e. membrane biological reactors) are proved to significantly reduce the amount of these resistant elements in the RWW (Munir et al., 2011). RWW was reported to contain tetracycline and sulfonamide resistant bacteria among others, as well as few dozens of clinically relevant ARGs, including genes conferring resistance to aminoglycosides,  $\beta$ -lactams, chloramphenicol, fluoroquinolones,

693 macrolides, rifampicin, tetracycline, trimethoprim, and sulfonamide antibiotics and other  
694 multidrug resistance genes (Szczepanowski et al. 2009). Moreover, the class 1 integron genes  
695 (*intI1*), often reported as a proxy of antibiotic resistance, was found in RWW (LaPara et al.,  
696 2011). The presence of ARB and ARGs in the agricultural environment receiving RWW and the  
697 potential implications that this phenomenon may pose to public health has recently attracted the  
698 attention of the scientific community (Holvoet et al., 2013; Broszat et al., 2014; Jechalke et al.,  
699 2015; Ben Said et al., 2016). Fahrenfeld et al. (2013) monitored the distribution of ARGs in the  
700 point of use of three RWW distribution systems in the western US and found that a broad  
701 spectrum of ARGs was present in the RWW passed through the distribution system, highlighting  
702 the importance of bacterial re-growth. The presence of *Lmip* and *gadAB* genes at the point of  
703 use of RWW distribution system also revealed the presence of the waterborne pathogens  
704 *Legionella pneumophila* (*Lmip*) and *Escherichia coli* (*gadAB*) in RWW. In addition, batch  
705 microcosm experiments revealed the presence of ARGs corresponding to resistance to  
706 sulfonamides (*sul1*, *sul2*) in soil following repeated irrigation with secondary-treated effluent  
707 (Fahrenfeld et al., 2013). With regard to the presence of ARB and ARGs in RWW-irrigated  
708 agricultural fields, results are controversial. Gatica and Cytryn (2013) reviewed recent studies  
709 that assessed the impact of RWW irrigation on antibiotic resistance in agricultural soils and  
710 concluded that RWW irrigation does not seem to impact antibiotic resistance levels in the soil  
711 microbiome. In addition, Negreanu et al. (2012) found identical or even lower levels of ARB  
712 and ARGs in agricultural soils irrigated with secondary-treated effluent for a prolonged period  
713 (6-18 years) in Israel compared with freshwater-irrigated soils. These findings suggest that  
714 antibiotic resistant elements released in RWW-irrigated soils are not able to compete or survive  
715 in the soil environment and that they do not significantly contribute ARGs to soil bacteria,  
716 corroborating to already reported existence of native AR in soil microbiome (D'Costa et al.,

2006). Worth noting, tetracycline and ciprofloxacin resistant bacteria were absent from the freshwater samples, whereas their abundance in the RWW applied for irrigation ranged between 50 and 450 and between 700 and 1100 CFU mL<sup>-1</sup> for tetracycline and ciprofloxacin, respectively (Negreanu et al., 2012). In contrast, higher diversity and increased abundance of various ARGs in soils of urban parks irrigated with RWW compared with freshwater irrigation or pristine soil were recently reported (Wang et al., 2014; Han et al., 2016). RWW irrigation of urban parks in Beijing, China, resulted in the increased abundance of *tetG*, *tetW*, *sul1*, and *sul2* ARGs (Wang et al., 2014). The integrase gene (*intI1*) was also detected in high abundance and had significant positive correlation with *tetG*, *sul1*, and *sul2* genes. Additionally, bacteria hosting *sul2* and *intI1* genes were related with bacteria, such as *Klebsiella oxytoca*, *Acinetobacter baumannii*, *Shigella flexneri*, whose potential to get in contact with humans may raise public health concerns (Wang et al., 2014). Han et al. (2016) reported that the ARGs detected in urban parks in Victoria, Australia, were significantly more abundant in RWW irrigated areas. Although the abundance of the genes *intI1* and the transposase *tnpA* were not significantly higher in RWW-irrigated urban parks compared with the non RWW-irrigated ones, the patterns of ARGs in both types of area were different, demonstrating that the impact of RWW irrigation was noticeable (Han et al., 2016). The overview of the studies described above, indicates that ARB and ARG dynamics along the RWW-soil-crop continuum are highly complex and that the persistence of ARB and the horizontal transfer of ARGs across these environmental barriers undoubtedly depend on a myriad of biotic and abiotic factors. The apparently controversial findings about the impacts in soil due to RWW irrigation may result, at least in part, to some practical and methodological limitations that can be illustrated based on some simple assumptions. One refers to the fact that soil contains a high abundance of bacteria, which means that even if RWW-derived bacteria accumulate in the soil, it may take several



741 decades to produce noticeable effects. One gram of bulk soil can contain  $10^8$  bacterial cells and  
742 more than  $10^4$  species (Raynaud and Nunan, 2014), while one mL of RWW may contain less  
743 than  $10^6$  bacterial cells, of which, in average, less than  $10^3$  host an acquired antibiotic resistance  
744 gene (Laht et al., 2014; Manaia et al., 2016). Assuming a soil water concentration of 10% (w/w),  
745 it could be estimated that the prevalence of acquired ARGs in soil would be of 0.0001%.  
746 Considering the unlikely scenario that due to aggregation or bacterial growth, the prevalence of  
747 that ARG increased 100 times, it would be 0.01%. This practical aspect stumbles on the second  
748 type of limitation, the methodological constraints. When DNA extracts, used for ARGs  
749 quantification, are prepared, normally from an amount equivalent to 0.25-1 g of soil, the ARGs  
750 that are being recovered are most probably close or below the quantification limit of most  
751 quantitative polymerase chain reaction (PCR) protocols commonly used and that ranges  $1:10^3$ -  
752  $10^6$  (ARGs:16S rRNA gene, referring to total bacteria) (Laht et al., 2014; Narciso-da-Rocha and  
753 Manaia, 2017). Moreover, since in the environment bacteria live mainly as aggregates, the  
754 quantification of ARGs may be also affected by random events. Hence, the heterogeneity of  
755 samples, not always overcome by technical replicates, and the aforementioned scarcity of ARGs,  
756 may lead to contradictory findings, as those reported above. Another approach that can be used  
757 to assess impacts of ARGs dissemination via RWW irrigation is based on metagenomic surveys.  
758 In metagenomics, as in quantitative PCR, the results are frequently expressed as a ratio between  
759 the gene of interest and a bacterial housekeeping gene, usually the 16S rRNA or, in alternative,  
760 to the total number of sequence reads (Graham et al., 2011; Christgen et al., 2015; Munck et al.,  
761 2015). Again, these values express a prevalence, which for the reasons evoked above may be too  
762 low to give an expressive result. It should be also noted that DNA may persist in soil for long  
763 periods of time and therefore targeting of ARGs using qPCR-based methods may in essence be

764 targeting relic DNA bound to clay particles and/or organic matter and not viable bacteria  
765 ([Becerra-Castro et al., 2015](#); [Carini et al., 2016](#)).

766  
767 However, the apparent inconsistency of the studies that aim to assess the potential impacts of the  
768 use of RWW for irrigation, should not be perceived as the absence of risk. A given ARB and  
769 ARG even at very low prevalence in a given environment may represent a high risk for the  
770 spreading of antibiotic resistance or for human health. It will contribute for the spreading of  
771 antibiotic resistance, if the ARB has the capacity to proliferate in the environment, is ubiquitous  
772 and harbors mobile genetic elements that can be transferred by horizontal gene transfer. In  
773 addition, an ARB and ARG will represent a threat for human health, if humans have a high  
774 exposure to places where the ARB is present (e.g. food crops cultivated in RWW-irrigated  
775 fields), if the ARB is able to colonize humans and, in the worst case, if the ARB contains also  
776 virulence factors ([Manaia, 2017](#)). In this aspect, it is worth mentioning that numerous  
777 wastewater ARB and ARGs are also potential human pathogens ([Vaz-Moreira et al., 2014](#)). For  
778 example, members of the families *Pseudomonadaceae*, *Burkholderiaceae* or *Moraxellaceae* that  
779 include opportunistic pathogens such as *Pseudomonas aeruginosa*, *Burkholderia cepacia* or  
780 *Acinetobacter baumannii*, are frequently detected in RWW and are also able to survive in soil  
781 and even to have an endophytic lifestyle in different crops ([Vaz-Moreira et al., 2014](#); [Becerra-](#)  
782 [Castro et al, 2015](#)).

783  
784  
785 **6. Uptake of antibiotics by reclaimed wastewater-irrigated crop plants in real and**  
786 **simulated field conditions**

787 Several classes of antibiotics have been proven to be taken up through roots and translocated to  
788 the aerial parts of crop plants grown under hydroponic or greenhouse control conditions, as well  
789 as in manure- and biosolids-amended and RWW-irrigated soils, in real agricultural systems  
790 (Boxall et al., 2012; Tanoue et al., 2012; Goldstein et al., 2014; Wu et al., 2015; Miller et al.,  
791 2016; Christou et al., 2017). Among them, chloramphenicol, sulfonamides, fluoroquinolones,  
792 and lincosamides are the ones with the highest bioconcentration factors (Pan et al., 2014).  
793 However, despite the relatively large number of predominantly descriptive studies undertaken in  
794 order to investigate root uptake of antibiotics, the mechanistic understanding of antibiotics  
795 uptake by crop plants remains rather limited (Miller et al., 2016). It has been previously shown  
796 that the uptake of antibiotics by crop plants is largely dependent on their  
797 bioavailability/bioaccessibility in soil pore water near the rhizosphere (sorption to soil  
798 constituents and transformation by soil organisms reduce bioavailability), and thus on their  
799 physicochemical properties and the properties of the soil environment (see Fig. 1) (Goldstein et  
800 al., 2014). Once taken up, the transport of antibiotics within the plant vascular translocation  
801 system (xylem and phloem) largely depends on their physicochemical properties (i.e.  
802 lipophilicity and electrical charge), as well as on the plant's physiology and transpiration rate  
803 (Goldstein et al., 2014; Dodgen et al., 2015) and environmental conditions (i.e. drought stress)  
804 (Zhang et al., 2016). Several antibiotics enter the root through the epidermis of growing root tips  
805 and subsequently pass through the cortex and the endodermis to reach the vascular tissues,  
806 where they can then be transported via the xylem to aboveground tissues. The movement of  
807 antibiotics from the soil pore water to the vascular tissues of plants may be distinguished to  
808 transmembrane, symplastic and apoplastic, depending on the ability of antibiotics to cross the  
809 membranes of plant cells (Miller et al., 2016). The presence of the Casparian strip in the  
810 endodermis which acts as a hydrophobic barrier between the apoplast and the vascular tissue,

811 suggests that antibiotics must at least once follow the symplastic route, which is constituted of  
812 selective binding sites and channels (Kong et al., 2007; Tanoue et al., 2012; Malchi et al., 2014).  
813 As a result, the lipophilicity and speciation of antibiotics strongly affects their root uptake by  
814 and translocation within the plants. The octanol-water partition coefficient ( $K_{ow}$ ) has been  
815 suggested as a predictor of uptake behavior of non ionizable organic compounds (Hsu et al.,  
816 1990). However, the movement of polar and ionizable antibiotics (the majority of antibiotics fall  
817 into this category) through plant cell membranes may be impeded by interactions with the  
818 negative surface potential of the cytoplasmic membrane (Trapp, 2004), by ion trapping, which is  
819 common for sulfonamides (Goldstein et al., 2014; Christou et al., 2016) and by sorption to plant  
820 cell walls (Trapp, 2004), making  $K_{ow}$  an inappropriate indicator for the estimation of ionizable  
821 antibiotics movement within and through plant cells. The pH-dependent speciation of ionic  
822 compounds ( $D_{ow}$ ) is considered to be a more appropriate descriptor for the ability of ionizable  
823 antibiotics to cross cell membranes and translocated within the plant than  $K_{ow}$  (Wu et al., 2013b;  
824 Hyland et al., 2015).

825  
826 The uptake and translocation of antibiotics within RWW-irrigated crop plants grown in real  
827 agricultural systems, where a cocktail of antibiotics occurs in RWW and the complexity of soil-  
828 plant-environment interactions prevails, has not been widely studied. Only few studies followed  
829 experimental setups where real RWW was applied for the irrigation of crop plants in field,  
830 representing actual farming practices, or genuine soil, or ecological conditions typical for  
831 commercial agriculture farming, simultaneously allowing for the assessment of the actual  
832 potential uptake of antibiotics by crops and its integration into a database for risk assessment  
833 (Malchi et al., 2014; Prosser and Sibley, 2015) (see Table 3). Wu et al. (2014) did not detect  
834 sulfamethoxazole and trimethoprim in plant tissues (root, leaf, stems, fruits) of vegetables

growing in field and irrigated with both tertiary-treated effluent or tertiary-treated effluent spiked with the two antibiotics and 17 other pharmaceuticals and personal care products at a concentration of 250 ng L<sup>-1</sup>, each. Lincomycin and ofloxacin antibiotics were detected in the leaves (0.12 and 0.10 µg kg<sup>-1</sup> wet weight, respectively) of *Eruca sativa* L. plants grown in soil in pots under greenhouse conditions and irrigated with water spiked with these antibiotics, based on the mean concentration of these antibiotics found in RWW and rivers in Italy (0.25 and 0.15 µg L<sup>-1</sup>, respectively) (Marsoni et al., 2014). However, these antibiotics were not detected in the grains of *Zea mays* L. grown under similar experimental set up and conditions (Marsoni et al., 2014). Moreover, sulfamethoxazole and sulfapyridine was not detected in tomato and cucumber fruits from plants grown in different types of soils (sand, aeolian, alluvial) under greenhouse conditions and irrigated with RWW (the mean concentrations of sulfamethoxazole and sulfapyridine was 0.28 and 0.17 µg L<sup>-1</sup>, respectively), whereas sulfamethoxazole was found in the leaves of tomato plants grown in all three soil types and in the leaves of cucumber grown in sand, suggesting that sulfamethoxazole is preferentially transported in the xylem rather than in the phloem of tomatoes and cucumber plants (Goldstein et al., 2014). Sulfamethoxazole was also found in the roots (edible parts) of carrots and sweet potatoes (0.05-0.24 µg kg<sup>-1</sup> wet weight) grown in soil in lysimeters and irrigated with RWW provided by a conventional activated-sludge wastewater treatment facility (the mean concentration of sulfamethoxazole in RWW was 0.05 µg L<sup>-1</sup>) (Malchi et al., 2014). Riemenschneider et al. (2016) detected ciprofloxacin in the edible tissues of cabbage and carrot (~5 and ~10 µg kg<sup>-1</sup> dry weight) grown in field and irrigated with water abstracted from the Zarqa River (the mean concentration of ciprofloxacin was 0.3 µg L<sup>-1</sup>), which is constituted of RWW from the largest WWTP in Jordan (As Samra WWTP, activated sludge and chlorination) as the main component and spring and runoff water, in Jordan.

859

860 By conducting a field study, [Christou et al. \(2017\)](#) explored the long-term (three years) effects of  
861 two distinctly tertiary-treated effluents (effluent from WWTP applying activated sludge, slow  
862 sand filtration and chlorination, and effluent derived from an MBR treatment) applied for the  
863 irrigation of tomato plants under commercial agricultural farming on the fate of  
864 sulfamethoxazole and trimethoprim in soil and their uptake and bioaccumulation in tomato  
865 fruits. The concentration of these antibiotics was determined in fruits harvested at the end of the  
866 harvesting period (last harvest) for the first two years of the study, while at the third year of the  
867 study antibiotics' concentrations were determined at fruits harvested at the beginning (first  
868 harvest), middle (fourth harvest) and the end of the harvesting period (seventh harvest) (seven to  
869 eight harvests took place in each year of the study). Results revealed that the concentration of  
870 these antibiotics in both the soil and tomato fruits varied depending on the qualitative  
871 characteristics of the RWW applied for irrigation and the duration of irrigation. The  
872 concentration of both antibiotics in fruits increased with the increasing duration of RWW  
873 irrigation, reaching the highest concentration values during the last harvest of the third year of  
874 the study ( $5.26 \mu\text{g kg}^{-1}$  for sulfamethoxazole and  $3.40 \mu\text{g kg}^{-1}$  for trimethoprim; in dry weight  
875 basis); the bioconcentration factor of sulfamethoxazole and trimethoprim reached its highest  
876 values during the last harvest of the third year of the study (5.42 and 6.44, respectively)  
877 ([Christou et al., 2017](#)).

878

879 The uptake of sulfamethoxazole, trimethoprim and ofloxacin by wheat plants (*Triticum aestivum*  
880 L.) grown in field and spray irrigated with RWW (effluent from WWTP where activated sludge  
881 and trickling filters were applied for treatment and disinfection) was evaluated at harvest, as well  
882 as three weeks before harvest, by [Franklin et al. \(2016\)](#). Straw and grain samples were rinsed

883 with methanol prior to the extraction and analysis of antibiotics in order to remove chemical  
884 compounds adhering to the outer surfaces, simultaneously allowing for the estimation of  
885 antibiotics within these tissues, as well as on their surfaces. Residues of each compound were  
886 present on most plant surfaces. Ofloxacin was found throughout the plant, with higher  
887 concentrations in the straw ( $10.2 \pm 7.05 \mu\text{g kg}^{-1}$ ) and lower concentrations in the grain ( $2.28 \pm 0.89$   
888  $\mu\text{g kg}^{-1}$ ). Trimethoprim was found only on the surfaces of grain ( $5.15 \pm 2.79 \mu\text{g kg}^{-1}$ ) and straw  
889 ( $1.1 \pm 0.54 \mu\text{g kg}^{-1}$ ), whereas sulfamethoxazole was concentrated within the grain ( $0.64 \pm 0.37 \mu\text{g}$   
890  $\text{kg}^{-1}$ ) (Franklin et al., 2016).

891

892 The above low volume of literature indicates that antibiotic uptake, translocation and  
893 accumulation in the edible parts of crop plants irrigated with RWW under real agricultural  
894 systems is feasible and likely dependent on crop species, soil type and soil pore water chemistry,  
895 the physicochemical properties of antibiotics, the concentration of antibiotics in RWW applied  
896 for irrigation and the duration that RWW irrigation is being practiced. Nonetheless, plenty of  
897 knowledge gaps still exist, requiring further studies utilizing RWW irrigation under field  
898 conditions. Such studies should incorporate a wider spectrum of plant species, while the  
899 concentration of antibiotics in RWW applied for irrigation, the soil and the edible parts of plants  
900 should be quantified, allowing for more accurate estimations of the bioconcentration factors and  
901 the estimation of potential public health risk associated with the consumption of such produce.  
902 The metabolites of antibiotics in plant tissues should also be quantified in studies evaluating the  
903 uptake of antibiotics by RWW-irrigated plants, since metabolites may occur in concentrations  
904 similar or even higher compared with the ones of parent compounds, while also being more  
905 toxic (Malchi et al., 2014; Miller et al., 2016; Paltiel et al., 2016). The potential uptake of ARB  
906 and ARGs by RWW-irrigated crop plants under real agricultural systems and their subsequent

entry into the food web with serious human health implications is not yet systematically evaluated, despite the fact that these antibiotic related contaminants are continuously released in agricultural soils due to the use of RWW for irrigation (Munir et al., 2011; Fahrenfeld et al., 2013). At the same time, studies revealed the potential internalization of ARB and ARGs by plants irrigated with RWW or grown in antibiotic polluted soil under controlled greenhouse conditions (Ye et al., 2016; Zhang et al., 2016).

## 7. Phytotoxic effects

Exposure to antibiotics has been shown to exert significant effects on plant development and physiology, such as lower rates of germination, inhibition of growth, tissues deformation, reduced photosynthetic rate and chlorophyll content, disturbances in redox homeostasis and other stress-related phenomena (Michelini et al., 2013; Bártíková et al., 2016; Christou et al., 2016) (see Fig. 3). With regard to model organisms, a study with *Arabidopsis* indicated that chlortetracycline interfered with plant calcium homeostasis, thereby causing severe stress symptoms in both roots and shoots (O'Connor and Aga, 2007). Using hairy root cultures of *Helianthus annuus*, the direct involvement of stress mediated reactive oxygen species (ROS) in oxytetracyclin degradation could be proven (Bruzzoniti et al., 2014). However, the majority of studies uncovering the adverse effects of antibiotic exposure to plants were conducted under hydroponic experimental set up in laboratory conditions using unrealistic antibiotic exposure concentrations (Migliore et al., 2003; Kong et al., 2007; Farkas et al., 2009; Xie et al., 2010; Hillis et al., 2011; Li et al., 2011b; Michelini et al., 2013; Pan and Chu, 2016b). Fewer studies have been conducted in soil under control greenhouse or field conditions by using slurries and manures (Migliore et al., 2010), or by spiking the soil with the studied antibiotics (Liu et al.,



2009). Marsoni et al. (2014) found no effects of lincomycin and ofloxacin on *Eruca sativa* L. and *Zea mays* L. grown in soil in greenhouse when applied through irrigation at concentrations of 1x, 10x and 100x of these antibiotics found in Italian RWW and rivers (0.15 and 0.25  $\mu\text{g L}^{-1}$ , respectively). Christou et al. (2016) monitored the phytotoxic effects of sulfamethoxazole and trimethoprim in alfalfa plants grown in sand and irrigated for 50 days with nutrient solution spiked with the targeted antibiotics at environmentally relevant concentrations (10  $\mu\text{g L}^{-1}$ ) and found that stress-related effects, manifested via membrane lipid peroxidation and oxidative burst, were local and confined to the roots rather than systemically to shoots and leaves, and exacerbated when the tested antibiotics were applied in mixture. Moreover, Christou et al. (2016), uncovered the role of both  $\text{H}_2\text{O}_2$  and NO in signal transduction for the orchestration of the detoxification mechanisms (induced antioxidant armory, induced expression of glutathione S-transferases; GSTs) in the leaves of alfalfa plants exposed to sulfamethoxazole and trimethoprim, as well as the involvement of proton pumps ( $\text{H}^+$ -ATPase) and cytochrome c oxidase (Cyt cOx) towards the detoxification of these antibiotics (Fig. 3). Overall, phytotoxicity is greatly dependent on factors including the compounds' properties and concentration in soil pore water, sorption kinetics, soil organic matter and pH, compound biodegradation rate, and the presence of other compounds in the soil.

## 8. Public health implications / Risk assessment

The antibiotic-mediated risks to human health associated with the consumption of agricultural produce from RWW-irrigated crops may be assessed either by estimating the daily or annual exposure of humans to antibiotics and converting it to medical dose equivalent (Marsoni et al., 2014; Pan et al., 2014; Wu et al., 2014), or by following the threshold of toxicological concern

(TTC) (Malchi et al., 2014), or the hazard quotient approach (Prosser and Sibley, 2015). The risks to human health due to the intake of antibiotics with the consumption of RWW-irrigated vegetables were revealed to be negligible by using the medical equivalent dose, as the daily intake due to the entrance of antibiotics in the food chain was 10-200 folds lower than the medical dose (Marsoni et al., 2014; Wu et al., 2014). By applying the TTC and hazard quotient approaches, Christou et al. (2017) assessed the effects of the intake of sulfamethoxazole and trimethoprim to both adults and toddlers due to the consumption of tomato fruits harvested from plants irrigated with tertiary-treated effluents during three consecutive growing periods in field, and found that the consumption of these fruits does not pose a health threat, since the daily consumption of tomato fruits by an adult in order to reach the TTC with regard to sulfamethoxazole and trimethoprim were 363.3 and 596.6 kg, whereas the respective values for toddler were 62.3 and 102.3 kg, respectively (the values of hazard quotient were equal or lower than 0.015). Marsoni et al. (2014) stated that the potential adverse effects of antibiotics along the food chain should not be neglected. Special attention should be given to antibiotics with structural alert for potential genotoxicity and carcinogenicity (i.e. sulfapyridine, sulfamethoxazole and ciprofloxacin) when the TTC approach is used, as the TTC value to be used for assessing the associated risks to human health is low ( $2.5 \text{ ng kg body weight}^{-1} \text{ day}^{-1}$ ). In a recently published review article, Prosser and Sibley (2015) assessed the human health risks of antibiotics and other pharmaceuticals in plant tissues due to RWW irrigation (as well as biosolids and manure amendments) and found that the majority of individual pharmaceuticals in the edible tissue of plants due to RWW irrigation represent a *de minimis* risk to human health, although when assuming additivity, the mixture of pharmaceuticals could potentially present a hazard. This is not only true for the parent compounds as such, but also for the metabolites, especially in mixtures.

979

980 It is obvious from the above that further in-field studies need to be performed in order to obtain  
981 more solid information on the safety of RWW use for irrigation. Such studies should examine  
982 the effects of the mixture of antibiotics present in the treated flows used for irrigation, as well as  
983 the potential additivity or synergies of the mixture of antibiotics and heavy metals towards  
984 toxicity, as well as the toxicity of metabolites, some of which may be accumulated in greater  
985 concentrations and exert higher toxic effects compared with the parent compounds ([Prosser and](#)  
986 [Sibley, 2015; Christou et al., 2017](#)). Another type of risks and public health impacts associated  
987 with RWW-irrigation are the potential uptake of ARB and ARGs by plants via soil and their  
988 entry into the food chain. These are issues poorly understood, but the current knowledge cannot  
989 exclude the possibility that ARB thriving in the environment can be transmitted to humans  
990 ([Ashbolt et al., 2013; Manaia, 2017](#)). Even if at very low abundance these bacteria may be  
991 transmitted to humans, eventually in an asymptomatic long-term colonization, noticed only  
992 when for some reason the general health condition is compromised ([Manaia, 2017](#)). The  
993 assessment of these risks is still difficult to achieve, due to different types of limitation, for  
994 example: i) the technical shortcomings on the detection and quantification of ARB and ARG in  
995 environmental matrices described above; ii) the ignorance about the number of ARB that may be  
996 required to start a successful colonization in the human body; or iii) the scant information on the  
997 paths of dissemination and transmission from the environment to humans. All these are  
998 important limitations to establish adequate recommendations about maximum admissible  
999 threshold values or to define critical control points or critical sources for ARB dissemination.

1000

1001

1002 **9. Concluding remarks and recommendations for future research**

1003 The number of in-field studies aiming to examine the fate of antibiotics, ARB and ARGs in the  
1004 agricultural environment as a result of RWW use for irrigation is currently limited. Moreover,  
1005 comparison of the results among studies for reaching a more solid conclusion is rendered  
1006 difficult due to the variations in plant growth conditions, analytical methods, RWW variability,  
1007 species/cultivars studied, and data reporting methods (e.g. fresh vs dry weight), or due to  
1008 insufficient information given (plant, environmental, and soil properties, irrigation regime,  
1009 antibiotics concentration in RWW and soil, etc.) (Miller et al., 2016). Based on the knowledge  
1010 gaps identified, we attempt here to provide recommendations for future research and suggest  
1011 future directions:

- 1012 • Studies should preferably be conducted under field conditions with genuine fully  
1013 characterized soil, real RWW flows, and by following common agricultural practices.  
1014 Importantly, the data regarding the antibiotics concentration in RWW applied for irrigation  
1015 and in the soil, should be reported. Moreover, other data regarding (a) soil properties (e.g. the  
1016 historical data of the field, soil pH, texture, CEC, electrical conductivity, organic matter  
1017 content, nutrient concentration), (b) environmental conditions (e.g. temperature, humidity,  
1018 abiotic stresses that may prevail during the experimental period), (c) irrigation regime and (d)  
1019 agricultural practices undertaken, should also be reported. To this effect, apart from the  
1020 extensive depiction of RWW-associated treatments, appropriate control treatments should be  
1021 applied and fully described, as well. Control treatments may refer to the irrigation of plants  
1022 with the same irrigation system (i.e. sprinkler, drip, sub-irrigation) and the same volumes and  
1023 frequency as RWW-irrigated plants, with tubewell water or tap water in which the absence of  
1024 antibiotics is verified before their use.
- 1025 • The transformation products of antibiotics in RWW and in soil should be monitored. The  
1026 potential uptake of metabolites and in general of the TPs present in agricultural soils as a

1027 result of biotic and abiotic transformation by plants warrants further investigation.  
1028 Metabolites in plant tissues should also be monitored, since sometimes they may exceed the  
1029 concentration of the parent compounds and exert more acute toxicity.

- 1030 • Comprehensive field-scale and microcosm studies should be conducted using a combination  
1031 of culture-based and culture-independent analyses in order to measure the impacts in terms of  
1032 ARGs and MGEs abundance and patterns, generating a body of information that support the  
1033 assessment of potential risks of resistance propagation through the path wastewater-  
1034 agricultural soil-crops-humans.
- 1035 • Further in-field studies need to be performed in order to obtain more solid information on the  
1036 possible public health risks of RWW reuse for irrigation. Such studies should examine the  
1037 effects of the mixture of antibiotics present in the RWW used for irrigation, as well as the  
1038 potential additivity or synergies of the mixture of antibiotics and heavy metals towards  
1039 toxicity, as well as the toxicity of metabolites, some of which may be accumulated in greater  
1040 concentrations and exert higher toxic effects compared with the parent compounds ([Prosser](#)  
1041 [and Sibley, 2015; Christou et al., 2017](#)).
- 1042 • Phytotoxic and other stress-related phenomena induced in crop plants under field conditions  
1043 due to their exposure to antibiotics as a result of RWW irrigation, as well as the detoxification  
1044 and overall defense mechanisms induced in response to such exposure, merit further  
1045 investigation. Studies should be conducted in soils using RWW (mixture of pharmaceuticals),  
1046 where additive or synergistic effects may prevail towards phytotoxicity.
- 1047 • An important question still seeking answer is the potential effects of antibiotics uptake and  
1048 accumulation in crop plant tissues on crop yield and fruit quality characteristics  
1049 (marketability, taste, antioxidant activity, etc.).

- 1050 • The effects of processes taking place in rhizosphere (root exudates and rhizosphere  
1051 microbiota) on antibiotic uptake by plants merit further investigation. The uptake mechanisms  
1052 of ionizable antibiotics (the majority of antibiotics) should be further explored in order to  
1053 unravel passive and active (energy dependent via channels and transporters) uptake, as well as  
1054 the translocation and reallocation of antibiotics within plant tissues (leaves, fruits) through the  
1055 vascular tissues.
- 1056 • The lack of validated and standardized protocols of analysis for the detection and precise  
1057 quantification of antibiotics in all environmental matrices that can guarantee the quality of the  
1058 results obtained, along with the complexity of these matrices and the reported metabolism and  
1059 transformation of antibiotics within this matrices (formation of TPs), highlight the need for  
1060 the development of validated and standardized methods for specific environmental matrices.

1061

#### 1062 ***Acknowledgements***

1063 “The authors would like to acknowledge the COST Action ES1403 NEREUS “New and  
1064 emerging challenges and opportunities in wastewater reuse”, supported by COST (European  
1065 Cooperation in Science and Technology, [www.cost.eu](http://www.cost.eu)).”

1066

1067

1068

1069

1070

1071

1072

1073

1074

1075 **References**

- 1076 Agüera, A., Lambropoulou, D. (2016) New Challenges for the Analytical Evaluation of  
1077 Reclaimed Water and Reuse Applications. in: Wastewater Reuse and Current Challenges.  
1078 Fatta-Kassinos, D., Dionysiou, D.D., Kümmerer, K. (eds), pp. 7-48, Springer International  
1079 Publishing Switzerland.
- 1080 Alder, A.C., McArdell, C.S., Golet, E.M., Ibric, S., Molnar, E., Nipales, N.S., Giger, W. (2001)  
1081 Occurrence and Fate of Fluoroquinolone, Macrolide, and Sulfonamide Antibiotics during  
1082 Wastewater Treatment and in Ambient Waters in Switzerland. in: Pharmaceuticals and Care  
1083 Products in the Environment, pp. 56-69, American Chemical Society.
- 1084 Ashbolt, N.J., Amézquita, A., Backhaus, T., Borriello, P., Brandt, K.K., Collignon, P., Coors, A.,  
1085 Finley, R., Gaze, W.H., Heberer, T., Lawrence, J.R., Larsson, D.G., McEwen, S.A., Ryan,  
1086 J.J., Schönfeld, J., Silley, P., Snape, J.R., Van den Eede, C., Topp, E., 2013. Human Health  
1087 Risk Assessment (HHRA) for environmental development and transfer of antibiotic  
1088 resistance. Environ Health Perspect 121 (9), 993-1001.
- 1089 Azanu, D., Mortey, C., Darko, G., Weisser, J.J., Styriahave, B., Abaidoo, R.C., 2016. Uptake of  
1090 antibiotics from irrigation water by plants. Chemosphere 157, 107-114.
- 1091 Baguer, A.J., Jensen, J., Krogh, P.H., 2000. Effects of the antibiotics oxytetracycline and tylosin  
1092 on soil fauna. Chemosphere 40 (7), 751-757.
- 1093 Baquero, F., Martínez, J.-L., Cantón, R., 2008. Antibiotics and antibiotic resistance in water  
1094 environments. Current Opinion in Biotechnology 19 (3), 260-265.
- 1095 Bártíková, H., Podlipná, R., Skálová, L., 2016. Veterinary drugs in the environment and their  
1096 toxicity to plants. Chemosphere 144, 2290-2301.

- 1097 Becerra-Castro, C., Lopes, A.R., Vaz-Moreira, I., Silva, E.F., Manaia, C.M., Nunes, O.C., 2015.  
 1098 Wastewater reuse in irrigation: A microbiological perspective on implications in soil  
 1099 fertility and human and environmental health. *Environment International* 75, 117-135.
- 1100 Ben Said, L., Klibi, N., Dziri, R., Borgo, F., Boudabous, A., Ben Slama, K., Torres, C., 2016.  
 1101 Prevalence, antimicrobial resistance and genetic lineages of *Enterococcus* spp. from  
 1102 vegetable food, soil and irrigation water in farm environments in Tunisia. *Journal of the*  
 1103 *Science of Food and Agriculture* 96 (5), 1627-1633.
- 1104 Berendonk, T.U., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Burgmann,  
 1105 H., Sorum, H., Norstrom, M., Pons, M.-N., Kreuzinger, N., Huovinen, P., Stefani, S.,  
 1106 Schwartz, T., Kisand, V., Baquero, F., Martinez, J.L., 2015. Tackling antibiotic resistance:  
 1107 the environmental framework. *Nat Rev Micro* 13 (5), 310-317.
- 1108 Bernier, S.P., Létoffé, S., Delepierre, M., Ghigo, J.-M., 2011. Biogenic ammonia modifies  
 1109 antibiotic resistance at a distance in physically separated bacteria. *Molecular Microbiology*  
 1110 81, 705-716.
- 1111 Bixio, D., Thoeye, C., De Koning, J., Joksimovic, D., Savic, D., Wintgens, T., Melin, T., 2006.  
 1112 Wastewater reuse in Europe. *Desalination* 187 (1-3), 89-101.
- 1113 Bondarczuk, K., Markowicz, A., Piotrowska-Seget, Z., 2016. The urgent need for risk assessment  
 1114 on the antibiotic resistance spread via sewage sludge land application. *Environment*  
 1115 *International* 87, 49-55.
- 1116 Bondarenko, S., Gan, J., Ernst, F., Green, R., Baird, J., McCullough, M., 2012. Leaching of  
 1117 pharmaceuticals and personal care products in turfgrass soils during recycled water  
 1118 irrigation. *J Environ Qual* 41 (4), 1268-1274.



1119 Borgman, O., Chefetz, B., 2013. Combined effects of biosolids application and irrigation with  
 1120 reclaimed wastewater on transport of pharmaceutical compounds in arable soils. *Water Res*  
 1121 47 (10), 3431-3443.

1122 Boxall, A.B.A., 2004. The environmental side effects of medication. *EMBO Reports* 5 (12), 1110-  
 1123 1116.

1124 Boxall, A.B.A., Rudd, M.A., Brooks, B.W., Caldwell, D.J., Choi, K., Hickmann, S., Innes, E.,  
 1125 Ostapyk, K., Staveley, J.P., Verslycke, T., Ankley, G.T., Beazley, K.F., Belanger, S.E.,  
 1126 Berninger, J.P., Carriquiriborde, P., Coors, A., DeLeo, P.C., Dyer, S.D., Ericson, J.F.,  
 1127 Gagne, F., Giesy, J.P., Gouin, T., Hallstrom, L., Karlsson, M.V., Larsson, D.G.J.,  
 1128 Lazorchak, J.M., Mastrocco, F., McLaughlin, A., McMaster, M.E., Meyerhoff, R.D.,  
 1129 Moore, R., Parrott, J.L., Snape, J.R., Murray-Smith, R., Servos, M.R., Sibley, P.K., Straub,  
 1130 J.O., Szabo, N.D., Topp, E., Tetreault, G.R., Trudeau, V.L., Van Der Kraak, G., 2012.  
 1131 Pharmaceuticals and Personal Care Products in the Environment: What Are the Big  
 1132 Questions? *Environmental Health Perspectives* 120 (9), 1221-1229.

1133 Brandt, K.K., Amezquita, A., Backhaus, T., Boxall, A., Coors, A., Heberer, T., Lawrence, J.R.,  
 1134 Lazorchak, J., Schonfeld, J., Snape, J.R., Zhu, Y.-G., Topp, E., 2015. Ecotoxicological  
 1135 assessment of antibiotics: A call for improved consideration of microorganisms.  
 1136 *Environment International* 85, 189-205.

1137 Broszat, M., Nacke, H., Blasi, R., Siebe, C., Huebner, J., Daniel, R., Grohmann, E., 2014.  
 1138 Wastewater Irrigation Increases the Abundance of Potentially Harmful  
 1139 Gammaproteobacteria in Soils in Mezquital Valley, Mexico. *Applied and Environmental*  
 1140 *Microbiology* 80 (17), 5282-5291.

1141 Bruzzoniti, M.C., Checchini, L., De Carlo, R.M., Orlandini, S., Rivoira, L., Del Bubba, M., 2014.  
 1142 QuEChERS sample preparation for the determination of pesticides and other organic

1143 residues in environmental matrices: a critical review. *Analytical and Bioanalytical*  
 1144 *Chemistry* 406, 4089-4116.

1145 Bush, K., Courvalin, P., Dantas, G., Davies, J., Eisenstein, B., Huovinen, P., Jacoby, G.A.,  
 1146 Kishony, R., Kreiswirth, B.N., Kutter, E., Lerner, S.A., Levy, S., Lewis, K., Lomovskaya,  
 1147 O., Miller, J.H., Mobashery, S., Piddock, L.J.V., Projan, S., Thomas, C.M., Tomasz, A.,  
 1148 Tulkens, P.M., Walsh, T.R., Watson, J.D., Witkowski, J., Witte, W., Wright, G., Yeh, P.,  
 1149 Zgurskaya, H.I., 2011. Tackling antibiotic resistance. *Nat Rev Microbiol* 9 (12), 894-896.

1150 Calderón-Preciado, D., Jiménez-Cartagena, C., Matamoros, V., Bayona, J.M., 2011. Screening of  
 1151 47 organic microcontaminants in agricultural irrigation waters and their soil loading. *Water*  
 1152 *Research* 45 (1), 221-231.

1153 Carini, P., Marsden, P.J., Leff, J.W., Morgan, E.E., Strickland, M.S., Fierer, N., 2016. Relic DNA  
 1154 is abundant in soil and obscures estimates of soil microbial diversity. *Nat Microbiol* 2,  
 1155 16242.

1156 Center for Disease Dynamics, Economics & Policy, 2015. State of the World's Antibiotics, 2015.  
 1157 CDDEP: Washington, D.C.

1158 Chefetz, B., Mualem, T., Ben-Ari, J., 2008. Sorption and mobility of pharmaceutical compounds  
 1159 in soil irrigated with reclaimed wastewater. *Chemosphere* 73 (8), 1335-1343.

1160 Christgen, B., Yang, Y., Ahammad, S.Z., Li, B., Rodriquez, D.C., Zhang, T., Graham, D.W.,  
 1161 2015. Metagenomics shows that low-energy anaerobic-aerobic treatment reactors reduce  
 1162 antibiotic resistance gene levels from domestic wastewater *Environmental Science &*  
 1163 *Technology* 49 (4), 2577-2584.

1164 Christou, A., Antoniou, C., Christodoulou, C., Hapeshi, E., Stavrou, I., Michael, C., Fatta-  
 1165 Kassinos, D., Fotopoulos, V., 2016. Stress-related phenomena and detoxification

1166 mechanisms induced by common pharmaceuticals in alfalfa (*Medicago sativa* L.) plants.  
 1167 Science of The Total Environment 557-558, 652-664.

1168 Christou, A., Karaolia, P., Hapeshi, E., Michael, C., Fatta-Kassinos, D., 2017. Long-term  
 1169 wastewater irrigation of vegetables in real agricultural systems: Concentration of  
 1170 pharmaceuticals in soil, uptake and bioaccumulation in tomato fruits and human health risk  
 1171 assessment. Water Research 109, 24-34.

1172 D'Costa, V.M., McGrann, K.M., Hughes, D.W., Wright, G.D., 2006. Sampling the antibiotic  
 1173 resistome. Science 311 (5759), 374-377.

1174 da Costa, P.M., Vaz-Piresa, P., Bernardoc, F., 2006. Antimicrobial resistance in *Enterococcus*  
 1175 *spp.* isolated in inflow, effluent and sludge from municipal sewage water treatment plants.  
 1176 Water Research 40 (8), 1735-1740.

1177 Dalkmann, P., Broszat, M., Siebe, C., Willaschek, E., Sakinc, T., Huebner, J., Amelung, W.,  
 1178 Grohmann, E., Siemens, J., 2012. Accumulation of Pharmaceuticals, *Enterococcus*, and  
 1179 Resistance Genes in Soils Irrigated with Wastewater for Zero to 100 Years in Central  
 1180 Mexico. PLoS ONE 7 (9), e45397.

1181 Davis, J.G., Truman, C.C., Kim, S.C., Ascough, J.C., Carlson, K., 2006. Antibiotic Transport via  
 1182 Runoff and Soil Loss. Journal of Environmental Quality 35 (6), 2250-2260.

1183 Ding, H., Wu, Y., Zou, B., Lou, Q., Zhang, W., Zhong, J., Lu, L., Dai, G., 2016. Simultaneous  
 1184 removal and degradation characteristics of sulfonamide, tetracycline, and quinolone  
 1185 antibiotics by laccase-mediated oxidation coupled with soil adsorption. Journal of  
 1186 Hazardous Materials 307, 350-358.

1187 Diwan, V., Stalsby Lundborg, C., Tamhankar, A.J., 2013. Seasonal and Temporal Variation in  
 1188 Release of Antibiotics in Hospital Wastewater: Estimation Using Continuous and Grab  
 1189 Sampling. PLoS ONE 8 (7), e68715.

1190 Dodgen, L.K., Ueda, A., Wu, X., Parker, D.R., Gan, J., 2015. Effect of transpiration on plant  
 1191 accumulation and translocation of PPCP/EDCs. *Environmental Pollution* 198 (0), 144-153.  
 1192 Durán-Álvarez, J.C., Jiménez-Cisneros, B. (2014) Beneficial and Negative Impacts on Soil by the  
 1193 Reuse of Treated/Untreated Municipal Wastewater for Agricultural Irrigation – A Review of  
 1194 the Current Knowledge and Future Perspectives. in: *Environmental Risk Assessment of Soil*  
 1195 *Contamination*. Hernandez-Soriano, M.C. (ed), pp. 137-197, InTech.  
 1196 Fahrenfeld, N.L., Ma, Y., O'Brien, M., Pruden, A., 2013. Reclaimed water as a reservoir of  
 1197 antibiotic resistance genes: distribution system and irrigation implications. *Frontiers in*  
 1198 *Microbiology* 4.  
 1199 Farkas, M.H., Mojica, E.-R.E., Patel, M., Aga, D.S., Berry, J.O., 2009. Development of a rapid  
 1200 biolistic assay to determine changes in relative levels of intracellular calcium in leaves  
 1201 following tetracycline uptake by pinto bean plants. *Analyst* 134 (8), 1594-1600.  
 1202 Farouk, F., Azzazy, H.M.E., Niessen, W.M.A., 2015. Challenges in the determination of  
 1203 aminoglycoside antibiotics, a review. *Analytica Chimica Acta* 890, 21-43.  
 1204 Fatta-Kassinos, D., Kalavrouziotis, I.K., Koukoulakis, P.H., Vasquez, M.I., 2011a. The risks  
 1205 associated with wastewater reuse and xenobiotics in the agroecological environment.  
 1206 *Science of The Total Environment* 409 (19), 3555-3563.  
 1207 Fatta-Kassinos, D., M.I., V., Kümmerer, K., 2011b. Transformation products of pharmaceuticals  
 1208 in surface waters and wastewater formed during photolysis and advanced oxidation  
 1209 processes - Degradation, elucidation of byproducts and assessment of their biological  
 1210 potency. *Chemosphere* 85 (5), 693-709.  
 1211 Ferhi, S., Bourdat-Deschamps, M., Daudin, J., Houot, S., Nélieu, S., 2016. Factors influencing the  
 1212 extraction of pharmaceuticals from sewage sludge and soil: an experimental design  
 1213 approach. *Analytical and Bioanalytical Chemistry* 408, 6153-6168.

1214 Franklin, A.M., Williams, C.F., Andrews, D.M., Woodward, E.E., Watson, J.E., 2016. Uptake of  
 1215 Three Antibiotics and an Antiepileptic Drug by Wheat Crops Spray Irrigated with  
 1216 Wastewater Treatment Plant Effluent. *J Environ Qual* 45 (2), 546-554.

1217 Ganiyu, S.O., van Hullebusch, E.D., Cretin, M., Esposito, G., Oturan, M.A., 2015. Coupling of  
 1218 membrane filtration and advanced oxidation processes for removal of pharmaceutical  
 1219 residues: A critical review. *Separation and Purification Technology* 156, Part 3, 891-914.

1220 Gao, P., Munir, M., Xagorarakis, I., 2012. Correlation of tetracycline and sulfonamide antibiotics  
 1221 with corresponding resistance genes and resistant bacteria in a conventional municipal  
 1222 wastewater treatment plant. *Sci Total Environ* 421-422, 173-183.

1223 Gatica, J., Cytryn, E., 2013. Impact of treated wastewater irrigation on antibiotic resistance in the  
 1224 soil microbiome. *Environmental Science And Pollution Research International* 20 (6), 3529-  
 1225 3538.

1226 Ghattas, A.-K., Fischer, F., Wick, A., Ternes, T.A., 2017. Anaerobic biodegradation of  
 1227 (emerging) organic contaminants in the aquatic environment. *Water Research* 116, 268-295.

1228 Goldstein, M., Shenker, M., Chefetz, B., 2014. Insights into the Uptake Processes of Wastewater-  
 1229 Borne Pharmaceuticals by Vegetables. *Environmental Science & Technology* 48 (10), 5593-  
 1230 5600.

1231 Gottschall, N., Topp, E., Metcalfe, C., Edwards, M., Payne, M., Kleywegt, S., Russell, P., Lapen,  
 1232 D.R., 2012. Pharmaceutical and personal care products in groundwater, subsurface drainage,  
 1233 soil, and wheat grain, following a high single application of municipal biosolids to a field.  
 1234 *Chemosphere* 87 (2), 194-203.

1235 Graham, D.W., Olivares-Rieumont, S., Knapp, C.W., Lima, L., Werner, D., Bowen, E., 2011.  
 1236 Antibiotic resistance gene abundances associated with waste discharges to the Almendares  
 1237 River near Havana, Cuba. *Environ Sci Technol* 45 (2), 418-424.

- 1238 Grossberger, A., Hadar, Y., Borch, T., Chefetz, B., 2014. Biodegradability of pharmaceutical  
1239 compounds in agricultural soils irrigated with treated wastewater. *Environmental Pollution*  
1240 185, 168-177.
- 1241 Gu, C., Karthikeyan, K.G., Sibley, S.D., Pedersen, J.A., 2007. Complexation of the antibiotic  
1242 tetracycline with humic acid. *Chemosphere* 66 (8), 1494-1501.
- 1243 Haham, H., Oren, A., Chefetz, B., 2012. Insight into the Role of Dissolved Organic Matter in  
1244 Sorption of Sulfapyridine by Semiarid Soils. *Environmental Science & Technology* 46 (21),  
1245 11870-11877.
- 1246 Halling-Sørensen, B., Sengeløv, G., Ingerslev, F., Jensen, B.L., 2003. Reduced Antimicrobial  
1247 Potencies of Oxytetracycline, Tylosin, Sulfadiazin, Streptomycin, Ciprofloxacin, and  
1248 Olaquinox Due to Environmental Processes. *Archives of Environmental Contamination*  
1249 and Toxicology 44 (1), 0007-0016.
- 1250 Hamilton, A.J., Stagnitti, F., Xiong, X., Kreidl, S.L., Benke, K.K., Maher, P., 2007. Wastewater  
1251 irrigation: the state of play. *Vadose Zone Journal* 6 (4), 823-840.
- 1252 Han, X.-M., Hu, H.-W., Shi, X.-Z., Wang, J.-T., Han, L.-L., Chen, D., He, J.-Z., 2016. Impacts of  
1253 reclaimed water irrigation on soil antibiotic resistome in urban parks of Victoria, Australia.  
1254 *Environmental Pollution* 211, 48-57.
- 1255 Hawker, D.W., Cropp, R., Boonsaner, M., 2013. Uptake of zwitterionic antibiotics by rice (*Oryza*  
1256 *sativa* L.) in contaminated soil. *Journal of Hazardous Material* 263, 458-466.
- 1257 Hebert, V.R., Miller, G.C., 1990. Depth dependence of direct and indirect photolysis on soil  
1258 surfaces. *Journal of Agricultural and Food Chemistry* 38 (3), 913-918.
- 1259 Hillis, D.G., Fletcher, J., Solomon, K.R., Sibley, P.K., 2011. Effects of Ten Antibiotics on Seed  
1260 Germination and Root Elongation in Three Plant Species. *Archives of Environmental*  
1261 *Contamination and Toxicology* 60 (2), 220-232.

- 1262 Hinsinger, P., Plassard, C., Tang, C., Jaillard, B.t., 2003. Origins of root-mediated pH changes in  
1263 the rhizosphere and their responses to environmental constraints: A review. *Plant and Soil*  
1264 248 (1), 43-59.
- 1265 Holvoet, K., Samplers, I., Callens, B., Dewulf, J., Uyttendaele, M., 2013. Moderate Prevalence of  
1266 Antimicrobial Resistance in *Escherichia coli* Isolates from Lettuce, Irrigation Water, and  
1267 Soil. *Applied and Environmental Microbiology* 79 (21), 6677-6683.
- 1268 Homem, V., Santos, L., 2011. Degradation and removal methods of antibiotics from aqueous  
1269 matrices β€“ A review. *Journal of Environmental Management* 92 (10), 2304-2347.
- 1270 Hsu, F.C., Marxmiller, R.L., Yang, A.Y.S., 1990. Study of Root Uptake and Xylem Translocation  
1271 of Cinmethylin and Related Compounds in Detopped Soybean Roots Using a Pressure  
1272 Chamber Technique. *Plant Physiology* 93 (4), 1573-1578.
- 1273 Hu, F., Bian, K., Liu, Y., Su, Y., Zhou, T., Song, X., He, L., 2014. Development of a modified  
1274 QUick, Easy, CHEap, Effective, Rugged and Safe method for the determination of multi-  
1275 class antimicrobials in vegetables by liquid chromatography tandem mass spectrometry.  
1276 *Journal of Chromatography A* 1368, 52-63.
- 1277 Huang, X., Liu, C., Li, K., Su, J., Zhu, G., Liu, L., 2015. Performance of vertical up-flow  
1278 constructed wetlands on swine wastewater containing tetracyclines and tet genes. *Water Res*  
1279 70, 109-117.
- 1280 Hurtado, C., Domínguez, C., Pérez-Babace, L., Cañameras, N., Comas, J., Bayona, J.M., 2016.  
1281 Estimate of uptake and translocation of emerging organic contaminants from irrigation  
1282 water concentration in lettuce grown under controlled conditions. *Journal of Hazardous*  
1283 *Materials* 305, 139-148.
- 1284 Hyland, K.C., Blaine, A.C., Higgins, C.P., 2015. Accumulation of contaminants of emerging  
1285 concern in food crops—part 2: Plant distribution. *Environ Toxicol Chem* 34, 2222-2230.

1286 Jacobsen, A.M., Halling-Sørensen, B., Ingerslev, F., Hansen, S.H., 2004. Simultaneous extraction  
1287 of tetracycline, macrolide and sulfonamide antibiotics from agricultural soils using  
1288 pressurised liquid extraction, followed by solid-phase extraction and liquid chromatography-  
1289 tandem mass spectrometry. *J Chromatogr A* 1038 (1-2), 157-170.

1290 Jechalke, S., Broszat, M., Lang, F., Siebe, C., Smalla, K., Grohmann, E., 2015. Effects of 100  
1291 years wastewater irrigation on resistance genes, class 1 integrons and IncP-1 plasmids in  
1292 Mexican soil. *Frontiers in Microbiology* 6 (163).

1293 Jechalke, S., Heuer, H., Siemens, J., Amelung, W., Smalla, K., 2014. Fate and effects of  
1294 veterinary antibiotics in soil. *Trends in Microbiology* 22 (9), 536-545.

1295 Johnson, T.A., Stedtfeld, R.D., Wang, Q., Cole, J.R., Hashsham, S.A., Looft, T., Zhu, Y.-G.,  
1296 Tiedje, J.M., 2016. Clusters of Antibiotic Resistance Genes Enriched Together Stay  
1297 Together in Swine Agriculture. *mBio* 7 (2).

1298 Ju, F., Li, B., Ma, L., Wang, Y., Huang, D., Zhang, T., 2016. Antibiotic resistance genes and  
1299 human bacterial pathogens: Co-occurrence, removal, and enrichment in municipal sewage  
1300 sludge digesters. *Water Research* 91, 1-10.

1301 Keiluweit, M., Bougoure, J.J., Nico, P.S., Pett-Ridge, J., Weber, P.K., Kleber, M., 2015. Mineral  
1302 protection of soil carbon counteracted by root exudates. *Nature Clim. Change* 5 (6), 588-  
1303 595.

1304 Kinney, C.A., Furlong, E.T., Werner, S.L., Cahill, J.D., 2006. Presence and distribution of  
1305 wastewater-derived pharmaceuticals in soil irrigated with reclaimed water. *Environmental*  
1306 *Toxicology and Chemistry* 25, 317-326.

1307 Koba, O., Golovko, O., Kodešová, R., Fér, M., Grabic, R., 2017. Antibiotics degradation in soil:  
1308 A case of clindamycin, trimethoprim, sulfamethoxazole and their transformation products.  
1309 *Environmental Pollution* 220, 1251-1263.



- 1310 Kodešová, R., Grabic, R., Kočárek, M., Klement, A., Golovko, O., Fér, M., Nikodem, A., Jakšík,  
1311 O., 2015. Pharmaceuticals' sorptions relative to properties of thirteen different soils. Science  
1312 of The Total Environment 511, 435-443.
- 1313 Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton,  
1314 H.T., 2002. Pharmaceuticals, Hormones, and Other Organic Wastewater Contaminants in  
1315 U.S. Streams, 1999-2000: A National Reconnaissance. Environmental Science &  
1316 Technology 36 (6), 1202-1211.
- 1317 Kong, W.D., Zhu, Y.G., Liang, Y.C., Zhang, J., Smith, F.A., Yang, M., 2007. Uptake of  
1318 oxytetracycline and its phytotoxicity to alfalfa (*Medicago sativa* L.). Environmental  
1319 Pollution 147 (1), 187-193.
- 1320 Kopmann, C., Jechalke, S., Rosendahl, I., Groeneweg, J., Krögerrecklenfort, E., Zimmerling, U.,  
1321 Weichelt, V., Siemens, J., Amelung, W., Heuer, H., Smalla, K., 2013. Abundance and  
1322 transferability of antibiotic resistance as related to the fate of sulfadiazine in maize  
1323 rhizosphere and bulk soil. FEMS Microbiology Ecology 83 (1), 125-134.
- 1324 Kulshrestha, P., Giese, R.F.J., Aga, D.S., 2004. Investigating the molecular interactions of  
1325 oxytetracycline in clay and organic matter: insights on factors affecting its mobility in soil.  
1326 Environ Sci Technol 38 (15), 4097-4105.
- 1327 Kurwadkar, S.T., Adams, C.D., Meyer, M.T., Kolpin, D.W., 2007. Effects of sorbate speciation  
1328 on sorption of selected sulfonamides in three loamy soils. Journal of Agricultural and Food  
1329 Chemistry 55 (4), 1370-1376.
- 1330 Laganà, A., Cavaliere, C., 2015. High-resolution mass spectrometry in food and environmental  
1331 analysis. Anal Bioanal Chem 407 (21), 6235-6236.
- 1332 Laht, M., Karkman, A., Voolaid, V., Ritz, C., Tenson, T., Virta, M., Kisand, V., 2014.  
1333 Abundances of Tetracycline, Sulphonamide and Beta-Lactam Antibiotic Resistance Genes

1334 in Conventional Wastewater Treatment Plants (WWTPs) with Different Waste Load. , (), . .  
 1335 PLoS ONE 9 (8), e103705.

1336 LaPara, T.M., Burch, T.R., McNamara, P.J., Tan, D.T., Yan, M., Eichmiller, J.J., 2011. Tertiary-  
 1337 Treated Municipal Wastewater is a Significant Point Source of Antibiotic Resistance Genes  
 1338 into Duluth-Superior Harbor. Environmental Science & Technology 45 (22), 9543-9549.

1339 Li, Y.W., Wu, X.L., Mo, C.H., Tai, Y.P., Huang, X.P., Xiang, L., 2011a. Investigation of  
 1340 sulfonamide, tetracycline, and quinolone antibiotics in vegetable farmland soil in the pearl  
 1341 river delta area, Southern China. Journal of Agriculture & Food Chemistry 59, 7268-7276.

1342 Li, Z.-j., Xie, X.-y., Zhang, S.-q., Liang, Y.-c., 2011b. Negative Effects of Oxytetracycline on  
 1343 Wheat (*Triticum aestivum* L.) Growth, Root Activity, Photosynthesis, and Chlorophyll  
 1344 Contents. Agricultural Sciences in China 10 (10), 1545-1553.

1345 Lin, H., Jin, D., Freitag, T.E., Sun, W., Yu, Q., Fu, J., Ma, J., 2016. A compositional shift in the  
 1346 soil microbiome induced by tetracycline, sulfamonomethoxine and ciprofloxacin entering a  
 1347 plant-soil system. Environmental Pollution 212, 440-448.

1348 Lin, K., Gan, J., 2011. Sorption and degradation of wastewater-associated non-steroidal anti-  
 1349 inflammatory drugs and antibiotics in soils. Chemosphere 83 (3), 240-246.

1350 Liu, F., Ying, G.-G., Yang, J.-F., Zhou, L.-J., Tao, R., Wang, L., Zhang, L.-J., Peng, P.-A., 2010.  
 1351 Dissipation of sulfamethoxazole, trimethoprim and tylosin in a soil under aerobic and  
 1352 anoxic conditions. Environmental Chemistry 7 (4), 370-376.

1353 Liu, F., Ying, G.G., Tao, R., Zhao, J.L., Yang, J.F., Zhao, L.F., 2009. Effects of six selected  
 1354 antibiotics on plant growth and soil microbial and enzymatic activities. Environmental  
 1355 Pollution 157 (5), 1636-1642.

1356 Lopes, A.R., Becerra-Castro, C., Vaz-Moreira, I., Silva, M.E.F., Nunes, O.C., Manaia, C.M.  
 1357 (2015) Irrigation with Treated Wastewater: Potential Impacts on Microbial Function and

1358 Diversity in Agricultural Soils. in: Wastewater Reuse and Current Challenges. Fatta-  
 1359 Kassinos, D., Dionysiou, D.D., Kummerer, K. (eds), pp. 105-128, Springer International  
 1360 Publishing, Cham.

1361 Luby, E., Ibekwe, A.M., Zilles, J., Pruden, A., 2016. Molecular methods for assessment of  
 1362 antibiotic resistance in agricultural ecosystems: Prospects and challenges. *J Environ Qual* 45  
 1363 (2), 441-453.

1364 Luo, Y., Guo, W., Ngo, H.H., Nghiem, L.D., Hai, F.I., Zhang, J., Liang, S., Wang, X.C., 2014. A  
 1365 review on the occurrence of micropollutants in the aquatic environment and their fate and  
 1366 removal during wastewater treatment. *Science of The Total Environment* 473-474 (0), 619-  
 1367 641.

1368 Ma, T., Pan, X., Chen, L.k., Liu, W., Christie, P., Luo, Y., Wu, L., 2016. Effects of different  
 1369 concentrations and application frequencies of oxytetracycline on soil enzyme activities and  
 1370 microbial community diversity. *European Journal of Soil Biology* 76, 53-60.

1371 Malchi, T., Maor, Y., Tadmor, G., Shenker, M., Chefetz, B., 2014. Irrigation of Root Vegetables  
 1372 with Treated Wastewater: Evaluating Uptake of Pharmaceuticals and the Associated Human  
 1373 Health Risks. *Environmental Science & Technology* 48 (16), 9325-9333.

1374 Manaia, C.M., 2017. Assessing the risk of antibiotic resistance transmission from the environment  
 1375 to humans: non-direct proportionality between abundance and risk. *Trends in Microbiology*  
 1376 25 (3), 173 - 181.

1377 Manaia, C.M., Macedo, G., Fatta-Kassinos, D., Nunes, O.C., 2016. Antibiotic resistance in urban  
 1378 aquatic environments: can it be controlled? *Applied Microbiology and Biotechnology* 100  
 1379 (4), 1543-1557.

1380 Marsoni, M., De Mattia, F., Labra, M., Bruno, A., Bracale, M., Vannini, C., 2014. Uptake and  
 1381 effects of a mixture of widely used therapeutic drugs in *Eruca sativa* L. and *Zea mays* L.  
 1382 plants. *Ecotoxicology and Environmental Safety* 108 (0), 52-57.

1383 Matamoros, V., Calderon-Preciado, D., Dominguez, C., Bayona, J.M., 2012. Analytical  
 1384 procedures for the determination of emerging organic contaminants in plant material: A  
 1385 review. *Analytica Chimica Acta* 722 (0), 8-20.

1386 Meffe, R., de Bustamante, I., 2014. Emerging organic contaminants in surface water and  
 1387 groundwater: A first overview of the situation in Italy. *Science of The Total Environment*  
 1388 481 (0), 280-295.

1389 Michael, I., Rizzo, L., McArdell, C.S., Manaia, C.M., Merlin, C., Schwartz, T., Dagot, C., Fatta-  
 1390 Kassinos, D., 2012. Urban wastewater treatment plants as hotspots for the release of  
 1391 antibiotics in the environment: A review. *Water Research* 47 (3), 957-995.

1392 Michelini, L., La Rocca, N., Rascio, N., Ghisi, R., 2013. Structural and functional alterations  
 1393 induced by two sulfonamide antibiotics on barley plants. *Plant Physiology and Biochemistry*  
 1394 67, 55-62.

1395 Migliore, L., Cozzolino, S., Fiori, M., 2003. Phytotoxicity to and uptake of enrofloxacin in crop  
 1396 plants. *Chemosphere* 52 (7), 1233-1244.

1397 Migliore, L., Godeas, F., De Filippis, S.P., Mantovi, P., Barchi, D., Testa, C., Rubattu, N.,  
 1398 Brambilla, G., 2010. Hormetic effect(s) of tetracyclines as environmental contaminant on  
 1399 *Zea mays*. *Environmental Pollution* 158 (1), 129-134.

1400 Miller, E.L., Nason, S.L., Karthikeyan, K.G., Pedersen, J.A., 2016. Root Uptake of  
 1401 Pharmaceuticals and Personal Care Product Ingredients. *Environmental Science &*  
 1402 *Technology* 50 (2), 525-541.

1403 Müller, T., Rosendahl, I., Focks, A., Siemens, J., Klasmeier, J., Matthies, M., 2013. Short-term  
 1404 extractability of sulfadiazine after application to soils. *Environmental Pollution* 172, 180-  
 1405 185.

1406 Munck, C., Albertsen, M., Telke, A., Ellabaan, M.M.H., Nielsen, P.H., Sommer, M.O.A., 2015.  
 1407 Limited dissemination of the wastewater treatment plant core resistome. *Nature*  
 1408 *Communications* 6, 8452.

1409 Munir, M., Wong, K., Xagorarakis, I., 2011. Release of antibiotic resistant bacteria and genes in  
 1410 the effluent and biosolids of five wastewater utilities in Michigan. *Water Research* 45 (2),  
 1411 681-693.

1412 Munir, M., Xagorarakis, I., 2011. Levels of Antibiotic Resistance Genes in Manure, Biosolids, and  
 1413 Fertilized Soil. *Journal of Environmental Quality* 40 (1), 248-255.

1414 Narciso-da-Rocha, C., Manaia, C.M., 2017. The influence of the autochthonous wastewater  
 1415 microbiota and gene host on the fate of invasive antibiotic resistance genes. *Sci Total*  
 1416 *Environ* 575, 932-940.

1417 Negreanu, Y., Pasternak, Z., Jurkevitch, E., Cytryn, E., 2012. Impact of Treated Wastewater  
 1418 Irrigation on Antibiotic Resistance in Agricultural Soils. *Environmental Science &*  
 1419 *Technology* 46 (9), 4800-4808.

1420 Nesme, J., Cecillon, S., Delmont, P., Monier, J.-M., Vogel, T., B. M., Simonet, P.,  
 1421 2014. Large-Scale Metagenomic-Based Study of Antibiotic Resistance in the Environment.  
 1422 *Current Biology* 24 (10), 1096-1100.

1423 Nesme, J., Simonet, P., 2015. The soil resistome: a critical review on antibiotic resistance origins,  
 1424 ecology and dissemination potential in telluric bacteria. *Environ Microbiol* 17, 913-930.

1425 O'Connor, S., Aga, D.S., 2007. Analysis of tetracycline antibiotics in soil: Advances in  
 1426 extraction, clean-up, and quantification. *Trends in Analytical Chemistry* 26 (6), 456-465.

1427 Paltiel, O., Fedorova, G., Tadmor, G., Kleinstern, G., Maor, Y., Chefetz, B., 2016. Human  
 1428 Exposure to Wastewater-Derived Pharmaceuticals in Fresh Produce: A Randomized  
 1429 Controlled Trial Focusing on Carbamazepine. *Environmental Science & Technology* 50 (8),  
 1430 4476-4482.

1431 Pan, M., Chu, L.M., 2016a. Adsorption and degradation of five selected antibiotics in agricultural  
 1432 soil. *Sci. Total Environ.* 545-546 (48-56), 48-56.

1433 Pan, M., Chu, L.M., 2016b. Phytotoxicity of veterinary antibiotics to seed germination and root  
 1434 elongation of crops. *Ecotoxicology and Environmental Safety* 126, 228-237.

1435 Pan, M., Wong, C.K.C., Chu, L.M., 2014. Distribution of Antibiotics in Wastewater-Irrigated  
 1436 Soils and Their Accumulation in Vegetable Crops in the Pearl River Delta, Southern China.  
 1437 *Journal of Agricultural and Food Chemistry* 62 (46), 11062-11069.

1438 Park, J.Y., Huwe, B., 2016. Effect of pH and soil structure on transport of sulfonamide antibiotics  
 1439 in agricultural soils. *Environmental Pollution* 213, 561-570.

1440 Pedersen, J.A., Yeager, M.A., Suffet, I.H., 2003. Xenobiotic Organic Compounds in Runoff from  
 1441 Fields Irrigated with Treated Wastewater. *Journal of Agricultural and Food Chemistry* 51  
 1442 (5), 1360-1372.

1443 Petrie, B., Barden, R., Kasprzyk-Hordern, B., 2015. A review on emerging contaminants in  
 1444 wastewaters and the environment: Current knowledge, understudied areas and  
 1445 recommendations for future monitoring. *Water Research* 72, 3-27.

1446 Pike, N., Kingcombe, R., 2009. Antibiotic treatment leads to the elimination of *Wolbachia*  
 1447 endosymbionts and sterility in the diplodiploid collembolan *Folsomia candida*. *BMC*  
 1448 *Biology* 7 (1), 54.

1449 Price, L.B., Koch, B.J., Hungate, B.A., 2015. Ominous projections for global antibiotic use in  
 1450 food-animal production. *Proceedings of the National Academy of Sciences of the United*  
 1451 *States of America* 112 (18), 5554-5555.

1452 Prosser, R.S., Sibley, P.K., 2015. Human health risk assessment of pharmaceuticals and personal  
 1453 care products in plant tissue due to biosolids and manure amendments, and wastewater  
 1454 irrigation. *Environment International* 75 (0), 223-233.

1455 Puckowski, A., Mioduszewska, K., Łukaszewicz, P., Borecka, M., Caban, M., Maszkowska, J.,  
 1456 Stepnowski, P., 2016. Bioaccumulation and analytics of pharmaceutical residues in the  
 1457 environment: A review. *Journal of Pharmaceutical and Biomedical Analysis* 127, 232-255.

1458 Rand-Weaver, M., Margiotta-Casaluci, L., Patel, A., Panter, G.H., Owen, S.F., Sumpter, J.P.,  
 1459 2013. The Read-Across Hypothesis and Environmental Risk Assessment of  
 1460 Pharmaceuticals. *Environmental Science & Technology* 47 (20), 11384-11395.

1461 Raynaud, X., Nunan, N., 2014. Spatial Ecology of Bacteria at the Microscale in Soil. *PLOS ONE*  
 1462 9 (1), e87217.

1463 Reichel, R., Radl, V., Rosendahl, I., Albert, A., Amelung, W., Schlöter, M., Thiele-Bruhn, S.,  
 1464 Soil microbial community responses to antibiotic-contaminated manure under different soil  
 1465 moisture regimes. *Applied Microbiology and Biotechnology* 98 (14), 6487-6495.

1466 Revitt, D.M., Balogh, T., Jones, H., 2015. Sorption behaviours and transport potentials for  
 1467 selected pharmaceuticals and triclosan in two sterilised soils. *Journal of Soils and Sediments*  
 1468 15 (3), 594-606.

1469 Riemenschneider, C., Al-Raggad, M., Moeder, M., Seiwert, B., Salameh, E., Reemtsma, T., 2016.  
 1470 Pharmaceuticals, Their Metabolites, and Other Polar Pollutants in Field-Grown Vegetables  
 1471 Irrigated with Treated Municipal Wastewater. *Journal of Agricultural and Food Chemistry*  
 1472 64 (29), 5784-5792.

1473 Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., Michael, I., Fatta-  
1474 Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant  
1475 bacteria and genes spread into the environment: A review. *Science of The Total*  
1476 *Environment* 447 (0), 345-360.

1477 Runnalls, T.J., Margiotta-Casaluci, L., Kugathas, S., Sumpter, J.P., 2010. Pharmaceuticals in the  
1478 Aquatic Environment: Steroids and Anti-Steroids as High Priorities for Research. *Human*  
1479 *and Ecological Risk Assessment: An International Journal* 16 (6), 1318-1338.

1480 Salvia, M.V., Vulliet, E., Wiest, L., Baudot, R., Cren-Olivé, C., 2012. Development of a multi-  
1481 residue method using acetonitrile-based extraction followed by liquid chromatography-  
1482 tandem mass spectrometry for the analysis of steroids and veterinary and human drugs at  
1483 trace levels in soil. *Journal of Chromatography A* 1245, 122-133.

1484 Schauss, K., Focks, A., Heuer, H., Kotzerke, A., Schmitt, H., Thiele-Bruhn, S.r., Smalla, K.,  
1485 Wilke, B.-M., Matthies, M., Amelung, W., Klasmeier, J.r., Schlöter, M., 2009. Analysis,  
1486 fate and effects of the antibiotic sulfadiazine in soil ecosystems. *TrAC Trends in Analytical*  
1487 *Chemistry* 28 (5), 612-618.

1488 Schlüsener, M.P., Bester, K., Spiteller, M., 2003. Determination of antibiotics such as macrolides,  
1489 ionophores and tiamulin in liquid manure by HPLC-MS/MS. *Anal Bioanal Chem* 375 (7),  
1490 942-947.

1491 Schmitt, H., Haapakangas, H., van Beelen, P., 2005. Effects of antibiotics on soil  
1492 microorganisms: time and nutrients influence pollution-induced community tolerance. *Soil*  
1493 *Biology and Biochemistry* 37 (10), 1882-1892.

1494 Seifrtová, M., Nováková, L., Lino, C., Pena, A., Solich, P., 2009. An overview of analytical  
1495 methodologies for the determination of antibiotics in environmental waters. *Analytica*  
1496 *Chimica Acta* 649 (2), 158-179.



1497 Senyuva, H.Z., Gökmen, V., Sarikaya, E.A., 2015. Future perspectives in Orbitrap™-high-  
 1498 resolution mass spectrometry in food analysis: a review. Food Additives and Contaminants -  
 1499 Part A Chemistry, Analysis, Control, Exposure and Risk Assessment 32 (10), 1568-1606.

1500 Sollic, M., Roy-Lachapelle, A., Gasser, M.-O., Coté, C., Généreux, M., Sauvé, S., 2016.  
 1501 Fractionation and analysis of veterinary antibiotics and their related degradation products in  
 1502 agricultural soils and drainage waters following swine manure amendment. Science of The  
 1503 Total Environment 543, Part A, 524-535.

1504 Szczepanowski, R., Linke, B., Krahn, I., Gartemann, K.-H., Gutzkow, T., Eichler, W., Puhler, A.,  
 1505 Schluter, A., 2009. Detection of 140 clinically relevant antibiotic-resistance genes in the  
 1506 plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility  
 1507 to selected antibiotics. Microbiology 155 (7), 2306-2319.

1508 Tanoue, R., Sato, Y., Motoyama, M., Nakagawa, S., Shinohara, R., Nomiya, K., 2012. Plant  
 1509 uptake of pharmaceutical chemicals detected in recycled organic manure and reclaimed  
 1510 wastewater. Journal of Agricultural and Food Chemistry 60 (41), 10203-10211.

1511 Ternes, T.A., Bonerz, M., Herrmann, N., Teiser, B., Andersen, H.R., 2007. Irrigation of treated  
 1512 wastewater in Braunschweig, Germany: an option to remove pharmaceuticals and musk  
 1513 fragrances. Chemosphere 66 (5), 894-904.

1514 Thiele-Bruhn, S., Seibicke, T., Schulten, H.-R., Leinweber, P., 2004. Sorption of Sulfonamide  
 1515 Pharmaceutical Antibiotics on Whole Soils and Particle-Size Fractions. Journal of  
 1516 Environmental Quality 33 (4), 1331-1342.

1517 Tong, L., Liu, H., Xie, C., Li, M., 2016. Quantitative analysis of antibiotics in aquifer sediments  
 1518 by liquid chromatography coupled to high resolution mass spectrometry. J Chromatogr A.  
 1519 1452, 58-66.

1520 Topp, E., Renaud, J., Sumarah, M., Sabourin, L., 2016. Reduced persistence of the macrolide  
 1521 antibiotics erythromycin, clarithromycin and azithromycin in agricultural soil following  
 1522 several years of exposure in the field. *Science of The Total Environment* 562, 136-144.

1523 Trapp, S., 2004. Plant uptake and transport models for neutral and ionic chemicals.  
 1524 *Environmental Science and Pollution Research* 11 (1), 33-39.

1525 Tulp, H.C., Goss, K.-U., Schwarzenbach, R.P., Fenner, K., 2008. Experimental determination of  
 1526 LSER parameters for a set of 76 diverse pesticides and pharmaceuticals. *Environmental*  
 1527 *Science & Technology* 42 (6), 2034-2040.

1528 Van Boeckel, T.P., Brower, C., Gilbert, M., Grenfell, B.T., Levin, S.A., Robinson, T.P., Teillant,  
 1529 A., Laxminarayan, R., 2015. Global trends in antimicrobial use in food animals.  
 1530 *Proceedings of the National Academy of Sciences* 112 (18), 5649-5654.

1531 Van Boeckel, T.P., Gandra, S., Ashok, A., Caudron, Q., Grenfell, B.T., Levin, S.A.,  
 1532 Laxminarayan, R., 2014. Global Antibiotic Consumption 2000 to 2010: An Analysis of  
 1533 National Pharmaceutical Sales Data. *The Lancet Infectious Diseases* 3099 (14), 1-9.

1534 Varela, A.R., Ferro, G., Vredenburg, J., Yanık, M., Vieira, L., Rizzo, L., Lameiras, C., Manaia,  
 1535 C.M., 2013. Vancomycin resistant enterococci: from the hospital effluent to the urban  
 1536 wastewater treatment plant. *Sci Total Environ* 450-451, 155-161.

1537 Varela, A.R., Macedo, G.N., Nunes, O.C., Manaia, C.M., 2015. Genetic characterization of  
 1538 fluoroquinolone resistant *Escherichia coli* from urban streams and municipal and hospital  
 1539 effluents. *FEMS Microbiol Ecol* 91 (5), pii: fiv015.

1540 Vasquez, M.I., Lambrianides, A., Schneider, M., Kümmerer, K., Fatta-Kassinos, D., 2014.  
 1541 Environmental side effects of pharmaceutical cocktails: What we know and what we should  
 1542 know. *Journal of Hazardous Materials* 279 (0), 169-189.

1543 Vasudevan, D., Bruland, G.L., Torrance, B.S., Upchurch, V.G., MacKay, A.A., 2009. pH-  
 1544 dependent ciprofloxacin sorption to soils: Interaction mechanisms and soil factors  
 1545 influencing sorption. *Geoderma* 151 (3), 68-76.

1546 Vaz-Moreira, I., Nunes, O.C., Manaia, C.M., 2014. Bacterial diversity and antibiotic resistance in  
 1547 water habitats: searching the links with the human microbiome. *FEMS Microbiology*  
 1548 *Reviews* 38 (4), 761-778.

1549 Walters, E., McClellan, K., Halden, R.U., 2010. Occurrence and loss over three years of 72  
 1550 pharmaceuticals and personal care products from biosolids-oil mixtures in outdoor  
 1551 mesocosms. *Water Research* 44 (20), 6011-6020.

1552 Wang, F.-H., Qiao, M., Lv, Z.-E., Guo, G.-X., Jia, Y., Su, Y.-H., Zhu, Y.-G., 2014. Impact of  
 1553 reclaimed water irrigation on antibiotic resistance in public parks, Beijing, China.  
 1554 *Environmental Pollution* 184, 247-253.

1555 Wardle, D.A., Bardgett, R.D., Klironomos, J.N., Setälä, H., van der Putten, W.H., Wall, D.H.,  
 1556 2004. Ecological Linkages Between Aboveground and Belowground Biota. *Science* 304  
 1557 (5677), 1629-1633.

1558 Wegst-Uhrich, S.R., Navarro, D.A., Zimmerman, L., Aga, D.S., 2014. Assessing antibiotic  
 1559 sorption in soil: a literature review and new case studies on sulfonamides and macrolides.  
 1560 *Chemistry Central Journal* 8 (1), 1-12.

1561 World Health Organization, 2014. Antimicrobial resistance: global report on surveillance.

1562 Wu, Q., Li, Z., Hong, H., Li, R., Jiang, W.-T., 2013a. Desorption of ciprofloxacin from clay  
 1563 mineral surfaces. *Water Research* 47 (1), 259-268.

1564 Wu, X., Conkle, J.L., Ernst, F., Gan, J., 2014. Treated Wastewater Irrigation: Uptake of  
 1565 Pharmaceutical and Personal Care Products by Common Vegetables under Field Conditions.  
 1566 *Environmental Science & Technology* 48 (19), 11286-11293.

1567 Wu, X., Dodgen, L.K., Conkle, J.L., Gan, J., 2015. Plant uptake of pharmaceutical and personal  
 1568 care products from recycled water and biosolids: a review. *Science of The Total*  
 1569 *Environment* 536, 655-666.

1570 Wu, X., Ernst, F., Conkle, J.L., Gan, J., 2013b. Comparative uptake and translocation of  
 1571 pharmaceutical and personal care products (PPCPs) by common vegetables. *Environment*  
 1572 *International* 60, 15-22.

1573 Wu, Y., Williams, M., Smith, L., Chen, D., Kookana, R., 2012. Dissipation of sulfamethoxazole  
 1574 and trimethoprim antibiotics from manure-amended soils. *J Environ Sci Health B.* 47 (4),  
 1575 240-249.

1576 Xie, X., Zhou, Q., He, Z., Bao, Y., 2010. Physiological and potential genetic toxicity of  
 1577 chlortetracycline as an emerging pollutant in wheat (*Triticum aestivum* L.). *Environmental*  
 1578 *Toxicology and Chemistry* 29, 922-928.

1579 Xu, J., Wu, L., Chang, A.C., 2009. Degradation and adsorption of selected pharmaceuticals and  
 1580 personal care products (PPCPs) in agricultural soils. *Chemosphere* 77 (10), 1299-1305.

1581 Yang, J.-F., Ying, G.-G., Yang, L.-H., Zhao, J.-L., Liu, F., Tao, R., Yu, Z.-Q., Peng, P.a., 2009.  
 1582 Degradation behavior of sulfadiazine in soils under different conditions. *Journal of*  
 1583 *Environmental Science and Health, Part B* 44 (3), 241-248.

1584 Ye, M., Sun, M., Feng, Y., Wan, J., Xie, S., Tian, D., Zhao, Y., Wu, J., Hu, F., Li, H., Jiang, X.,  
 1585 2016. Effect of biochar amendment on the control of soil sulfonamides, antibiotic-resistant  
 1586 bacteria, and gene enrichment in lettuce tissues. *Journal of Hazardous Materials* 309, 219-  
 1587 227.

1588 Yu, Z., Jiang, L., Yin, D., 2011. Behavior toxicity to *Caenorhabditis elegans* transferred to the  
 1589 progeny after exposure to sulfamethoxazole at environmentally relevant concentrations. *J*  
 1590 *Environ Sci (China)* 23 (2), 294-300.

1591 Zhang, Y.-L., Lin, S.-S., Dai, C.-M., Shi, L., Zhou, X.-F., 2014. Sorption-desorption and transport  
1592 of trimethoprim and sulfonamide antibiotics in agricultural soil: effect of soil type, dissolved  
1593 organic matter, and pH. *Environmental Science and Pollution Research* 21 (9), 5827-5835.

1594 Zhang, Y., Sallach, J.B., Hodges, L., Snow, D.D., Bartelt-Hunt, S.L., Eskridge, K.M., Li, X.,  
1595 2016. Effects of soil texture and drought stress on the uptake of antibiotics and the  
1596 internalization of Salmonella in lettuce following wastewater irrigation. *Environmental*  
1597 *Pollution* 208, Part B, 523-531.

1598  
1599  
1600  
1601  
1602  
1603  
1604  
1605  
1606  
1607  
1608  
1609  
1610  
1611  
1612  
1613  
1614

1615

1616

1617

1618 **Figure captions:**

1619 **Figure 1.** Main sources and fates of antibiotics in the agricultural environment receiving RWW for  
1620 irrigation. The reuse of RWW for irrigation constitutes a significant pathway for the introduction  
1621 of antibiotics to the agricultural environment, as conventional wastewater treatment processes are  
1622 only moderately effective at removing antibiotics from the RWW. Consequently, antibiotics are  
1623 routinely detected in RWW, and in RWW-irrigated agricultural soils and runoff from such sites,  
1624 and surface and groundwater systems and sediments receiving RWW. Antibiotics are taken up by,  
1625 and bioaccumulate in the edible tissues of RWW-irrigated crop plants, thus entering the food web  
1626 with potential negative implications to public health.

1627

1628 **Figure 2.** Examples of objectives and applications of approaches commonly used to detect and  
1629 quantify ARB and ARG in wastewater and in soil.

1630

1631 **Figure 3.** Following their uptake, translocation and bioaccumulation within crop plants, antibiotics  
1632 may exert phytotoxic and stress related phenomena in an organ-specific manner. Plants employ  
1633 sophisticated defense and detoxification mechanisms to overcome these adverse effects, with the  
1634 enhancement of the antioxidant defense system and the induction of glutathione S-transferases  
1635 (GSTs) and cytochrome P450 at the enzymatic and transcript level to be of high significance.  
1636 Antibiotics are also metabolized within the plant cells through oxidation, reduction and hydrolysis,  
1637 and through their conjugation with sugars and other macromolecules, which in turn facilitate their

1638 sequestration in the vacuole or their exclusion to the apoplast. Figure is modified from [Christou et](#)  
1639 [al. \(2016\)](#).

1640

1641

